NON-REDUCING SACCHARIDE-FORMING ENZYME, TREHALOSE-RELEASING ENZYME, AND PROCESS FOR PRODUCING SACCHARIDES USING THE ENZYMES

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending parent application no. 09/392,253, filed September 9, 1999, the entire contents of which being hereby incorporated herein by reference.

Background of the Invention

Field of the Invention

The present invention relates to a non-reducing saccharide-forming enzyme, a trehalose-releasing enzyme, and a process for producing saccharides using the enzymes.

Description of the Prior Art

Trehalose is a disaccharide consisting of two moles of glucose bound at their reducing residues, and is widely found in nature, for example, in microorganisms, fungi, algae, insects, *Crustacea*, etc. Since the saccharide has long been known as a useful saccharide substantially free of reducibility and having a satisfactory moisture-retaining action, it has been expected to use in extensive fields including foods, cosmetics, and pharmaceuticals. However, no efficient production of the saccharide was established, and this narrows the use of trehalose in spite of its outstanding expectation. Thus supply of trehalose in a lower cost is greatly expected.

As a proposal for such an expectation, the present inventors had already established a process for enzymatically producing trehalose from material starches through their energetic studies. The process is characterized by a step of subjecting reducing partial starch hydrolysates to the action of a non-reducing saccharide-forming enzyme, which forms a non-

reducing saccharide having a trehalose structure as an end unit from reducing partial starch hydrolysates, and to the action of a trehalose-releasing enzyme which acts on a non-reducing saccharide having a trehalose structure as an end unit in order to hydrolyze the site between a part of the trehalose structure and a part of the resting. These enzymes and processes thereof are disclosed in Japanese Patent Kokai Nos. 143,876/95, 213,283/95, 322,883/95, 298,880/95, 66,187/96, 66,188/96, 73,504/96, 84,586/96, and 336,388/96, applied for by the same applicant as the present invention. Thus, a low-cost production of trehalose was attained.

During the studies, they found an original finding that the non-reducing saccharide-forming enzyme can be applied for a novel production of non-reducing saccharides that can overcome conventional drawback residing in reducing partial starch hydrolysates. As a problem, reducing partial starch hydrolysates such as dextrins and maltooligosaccharides have advantageous features that they can be used as sweeteners and energy-supplementing saccharide sources, but as a demerit they are highly reactive with substances because of their reducibility and are susceptible to browning reaction when coexisted with amino acids and/or proteins and to readily deteriorate their quality. To overcome such a problem, it is only known a method to convert reducing partial starch hydrolysates into sugar alcohols using a high-pressure hydrogenation method, etc. In actual use, the method, however, needs much heats and instruments constructed under consideration

of safety in view of the use of hydrogen, resulting in a higher cost and much labor cost. On the contrary, the aforesaid non-reducing saccharide-forming enzyme as mentioned previously acts on reducing partial starch hydrolysates and forms non-reducing saccharide having a trehalose structure as an end unit, and the reaction proceeds under a relatively-mild condition due to its enzymatic reaction. Using the action of the enzyme, the present inventors established a novel efficient process for non-reducing saccharides using the enzyme, that can overcome conventional drawback residing in reducing partial starch hydrolysates. Because of these findings, the development of applicable uses for trehalose and non-reducing saccharides have become to be flourished in various fields, and this diversifies the uses of these saccharides and now remarkably increases the demands of the saccharides in a wide variety of fields.

Under these circumstances, a more efficient process for producing trehalose and non-reducing saccharides having a trehalose structure has been more expected in this art. A key to such an expectation is to establish a non-reducing saccharide-forming enzyme and a trehalose-releasing enzyme with various optimum conditions, and to provide a wide variety of sources for such enzymes usable in the production of the saccharides. Thus, an optimum enzyme can be chosen from various types of enzymes depending on the optimum conditions of another enzymes usable in combination with the above enzymes to produce the desired saccharides, as well as on installations and final uses of the saccharides produced, resulting in an efficient

production of the saccharides. Conventionally known non-reducing saccharide-forming enzymes can be grouped into those having optimum temperatures of relatively-lower temperatures of about 40°C or lower, and those having optimum temperatures of relatively-higher temperatures of about 60°C or higher. While conventionally known trehalose-releasing enzymes can be grouped into those having optimum temperatures in a relatively-lower temperature range, about 45°C or lower, and those having optimum temperatures in a relatively-higher temperature range, about 60°C or higher. However, any non-reducing saccharide-forming enzyme and a trehalose-releasing enzyme having an optimum temperature in a medium temperature range, about 50°C, have never yet been opened.

Among saccharide-related enzymes used in the production of saccharides from starch materials, enzymes as a major group have an optimum temperature in a medium temperature range. Such enzymes may be required in the process for producing the aforesaid trehalose and non-reducing saccharides; No non-reducing saccharide-forming enzyme and no trehalose-releasing enzyme, which have an optimum temperature in a medium temperature range, have not yet been established so that there has not yet been realized a process for producing saccharides in a sufficient yield using either or both of these enzymes together with the above saccharide-related enzymes. Depending on installations for producing saccharides and final uses of them, there have been required enzymes having an optimum temperature in a medium temperature range in their enzymatic

reactions. It is far from saying that it has established a process for producing saccharides in a satisfactorily-high yield using a non-reducing saccharide-forming enzyme and a trehalose-releasing enzyme. As described above the establishment of a non-reducing saccharide-forming enzyme and a trehalose-releasing enzyme having an optimum temperature in a medium temperature range, and a process for producing saccharides comprising non-reducing saccharides are in great demand.

Summary of the Invention

In view of this, the first object of the present invention is to provide a non-reducing saccharide-forming enzyme having an optimum temperature in a medium temperature range.

The second object of the present invention is to provide a DNA encoding the non-reducing saccharide-forming enzyme.

The third object of the present invention is to provide a process for producing the non-reducing saccharide-forming enzyme.

The fourth object of the present invention is to provide a trehalose-releasing enzyme having an optimum temperature in a medium temperature range.

The fifth object of the present invention is to provide a DNA encoding the trehalose-releasing enzyme.

The sixth object of the present invention is to provide a process for producing the trehalose-releasing enzyme.

The seventh object of the present invention is to provide a microorganism capable of producing the non-reducing saccharide-forming enzyme and/or the trehalose-releasing enzyme.

The eighth object of the present invention is to provide a process for producing saccharides comprising non-reducing saccharides, which uses the non-reducing saccharide-forming enzyme and/or the trehalose-releasing enzyme.

In order to attain the above objects, the present inventors extensively screened microorganisms, that can overcome the objects, in soils. As a result, they found that a microorganism newly isolated from a soil in Ako-shi, Hyogo, Japan, produced enzymes that can solve the above objects. The present inventors isolated separatory the desired non-reducing saccharide-forming enzyme and trehalose-releasing enzyme from the microorganism, and then identified their properties, revealing that the enzymes both had an optimum temperature in a medium temperature range. The identification of the microorganism confirmed that it was a novel microorganism of the genus *Arthrobacter*, and named *Arthrobacter* sp. S34. The microorganism was deposited on August 6, 1998, in the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, Higashi 1-1-3, Tsukuba-shi, Ibaraki, Japan, and accepted and has been maintained by the institute under the accession number of FERM BP-6450.

The present inventors continued studying, isolated DNAs encoding the above-identified enzymes from the

microorganism, *Arthrobacter* sp. S34, FERM BP-6450, decoded the nucleotide sequences, and determined the amino acid sequences of the enzymes. The inventors confirmed that *Arthrobacter* sp. S34, FERM BP-6450, and transformants, into which the DNAs obtained in the above had been introduced in a usual manner, produced desired amounts of enzymes. It was also confirmed that the enzymes thus obtained can be advantageously used in producing saccharides which comprise trehalose and non-reducing saccharides having a trehalose structure in a medium temperature range. The present invention was made based on these findings.

The first object of the present invention is solved by a novel non-reducing saccharide-forming enzyme that forms a non-reducing saccharide having a trehalose structure as an end unit from reducing partial starch hydrolysates, and has an optimum temperature in a medium temperature range.

The second object of the present invention is solved by a DNA encoding the non-reducing saccharide-forming enzyme.

The third object of the present invention is solved by a process for producing the non-reducing saccharide-forming enzyme, characterized in that it comprises the steps of culturing a microorganism capable of producing the enzyme, and collecting the produced enzyme from the culture.

The fourth object of the present invention is solved by a novel trehalose-releasing enzyme which specifically hydrolyses a non-reducing saccharide having a trehalose structure as an end unit and a glucose polymerization degree of

at least 3 at a site between a trehalose part and a part of the resting, and which has an optimum temperature in a medium temperature range.

The fifth object of the present invention is solved by a DNA encoding the trehalose-releasing enzyme.

The sixth object of the present invention is solved by a process for producing the trehalose-releasing enzyme, characterized in that it comprises the steps of culturing a microorganism capable of producing the enzyme, and collecting the produced enzyme from the culture.

The seventh object of the present invention is solved by a microorganism selected from *Arthrobacter* sp. S34, FERM BP-6450, and mutants thereof.

The eighth object of the present invention is solved by a process for producing saccharides, comprising the steps of allowing the either or both of the above enzymes to act on reducing partial starch hydrolysates to produce non-reducing saccharides, and collecting the non-reducing saccharides or saccharide compositions having a relatively-low reducibility and containing the non-reducing saccharides.

Brief Description of the Accompanying Drawings

FIG. 1 is a figure that shows the influence of temperature on the activity of a non-reducing saccharide-forming enzyme from *Arthrobacter* sp. S34, FERM BP-6450, according to the

present invention.

FIG. 2 is a figure that shows the influence of pH on the activity of a non-reducing saccharide-forming enzyme from *Arthrobacter* sp. S34, FERM BP-6450, according to the present invention.

FIG. 3 is a figure that shows the influence of temperature on the stability of a non-reducing saccharide-forming enzyme from *Arthrobacter* sp. S34, FERM BP-6450, according to the present invention.

FIG. 4 is a figure that shows the influence of pH on the stability of a non-reducing saccharide-forming enzyme from *Arthrobacter* sp. S34, FERM BP-6450, according to the present invention.

FIG. 5 is a restriction map of the recombinant DNA pGY1 according to the present invention. The bold line shows the nucleotide sequence from *Arthrobacter* sp. S34, FERM BP-6450. The black arrow within the bold line shows a nucleotide sequence encoding the present non-reducing saccharide-forming enzyme, while the oblique arrow shows a nucleotide sequence encoding the present trehalose-releasing enzyme.

FIG. 6 is a restriction map of the recombinant DNA pGY2 according to the present invention. The bold line shows the nucleotide sequence from *Arthrobacter* sp. S34, FERM BP-6450. The black arrow within the bold line shows a nucleotide sequence encoding the present non-reducing saccharide-forming enzyme.

FIG. 7 is a restriction map of the recombinant DNA

pGY3 according to the present invention. The black arrow shows the nucleotide sequence, encoding the present non-reducing saccharide-forming enzyme, from *Arthrobacter* sp. S34, FERM BP-6450.

FIG. 8 is a figure that shows the influence of temperature on the activity of a trehalose-releasing enzyme from *Arthrobacter* sp. S34, FERM BP-6450, according to the present invention.

FIG. 9 is a figure that shows the influence of pH on the activity of a trehalose-releasing enzyme from *Arthrobacter* sp. S34, FERM BP-6450, according to the present invention.

FIG. 10 is a figure that shows the influence of temperature on the stability of a trehalose-releasing enzyme from *Arthrobacter* sp. S34, FERM BP-6450, according to the present invention.

FIG. 11 is a figure that shows the influence of pH on the stability of a trehalose-releasing enzyme from *Arthrobacter* sp. S34, FERM BP-6450, according to the present invention.

FIG. 12 is a restriction map of the recombinant DNA pGZ2 according to the present invention. The bold line shows the nucleotide sequence from *Arthrobacter* sp. S34, FERM BP-6450. The oblique arrow within the bold line shows a nucleotide sequence encoding the present trehalose-releasing enzyme.

FIG. 13 is a restriction map of the recombinant DNA pGZ3 according to the present invention. The oblique arrow shows the nucleotide sequence from *Arthrobacter* sp. S34, FERM

Detailed Description of the Invention

The present invention relates to a non-reducing saccharide-forming enzyme and a trehalose-releasing enzyme, and a process for producing a saccharide using either or both of the enzymes. The wording "**non-reducing saccharide-forming enzyme**" as referred to in the present invention represents an enzyme which has an action of forming a non-reducing saccharide having a trehalose structure as an end unit from reducing partial starch hydrolysates. The wording "**trehalose-releasing enzyme**" as referred to in the present invention represents an enzyme which specifically hydrolyses a non-reducing saccharide having a trehalose structure as an end unit and a glucose polymerization degree of at least 3 at a site between the trehalose part and the resting part. The wording "a **medium temperature range**" as referred to in the present invention represents a middle temperature range in reaction temperatures which are conventionally used in producing saccharides from starch materials by an enzymatic reaction. In most cases of such processes, different reaction temperatures of about 10°C to about 100°C and around the temperatures are used. The non-reducing saccharide-forming enzyme according to the present invention has an action as such an enzyme and has an optimum temperature in a medium temperature range, preferably a temperature range over 40°C but less than 60°C, and more

preferably it has an optimum pH in an acid pH range in addition to the optimum temperature. The trehalose-releasing enzyme according to the present invention has an action as such an enzyme and has an optimum temperature in a medium temperature range, preferably a temperature range over 45°C but below 60°C, and more preferably it has an optimum pH in an acid pH range in addition to the optimum temperature. These present enzymes should not be restricted to their origins and sources.

The activity of the present non-reducing saccharide-forming enzyme is assayed as follows: One ml of an enzyme solution is added to four ml of 1.25 w/v % maltopentaose as a substrate in 20 mM phosphate buffer (pH 6.0), and the mixture solution is incubated at 50°C for 60 min. The reaction mixture is heated at 100°C for 10 min to suspend the enzymatic reaction, and the reaction mixture is precisely diluted by 10 times with deionized water, followed by determining the reducing power of the diluted solution on the Somogyi-Nelson's method. As a control, an enzyme solution, which had been heated at 100°C for 10 min to inactivate the enzyme, is treated similarly as above. One unit activity of the present enzyme is defined as the amount of enzyme which eliminates the reducing power of that of one μ mole of maltopentaose per minute when determined with the above-mentioned assay. The optimum temperature of the enzyme as referred to in the present invention is determined in accordance with the assay; It is assayed by adjusting the enzymatic reaction temperature at different temperatures including 50°C, allowing a prescribed amount of the enzyme to act on the

substrate at the different temperatures according to the assay, and determining the reduction level of reducing power at the temperatures in accordance with the assay, followed by comparing the determined reduction levels one another and determining the optimum temperature of the present enzyme that showed a maximum temperature.

The activity of the present trehalose-releasing enzyme is assayed as follows: One ml of an enzyme solution is added to four ml of 1.25 w/v % maltotriosyltrehalose, i.e., α-maltotetraosyl-α-D-glucoside, as a substrate, in 20 mM phosphate buffer (pH 6.0), and the mixture solution is incubated at 50°C for 30 min, followed by suspending the enzymatic reaction by the addition of the Somogyi copper solution and assaying the reducing power by the Somogyi-Nelson's method. As a control, it is similarly assayed using an enzyme solution which has been inactivated by heating at 100°C for 10 min. One unit activity of the present enzyme is defined as the amount of enzyme which increases the reducing power of one μ mole of glucose per minute when determined with the above-mentioned assay. The optimum temperature of the enzyme as referred to in the present invention is determined in accordance with the assay; It is assayed by adjusting the enzymatic reaction temperature at the different temperatures including 50°C, allowing a prescribed amount of the enzyme to act on the substrate at the temperatures according to the assay, and determining the increased level of reducing power at the different temperatures in accordance with the assay, followed by comparing the determined increased levels

one another and determining the optimum temperature of the present enzyme that showed a maximum temperature.

Explaining the present non-reducing saccharide-forming enzyme based on the amino acid sequence, the enzyme has the amino acid sequence of SEQ ID NO:1 as a whole, and has the amino acid sequences of SEQ ID NOs:2 to 6 as partial amino acid sequences in some cases. In addition to these enzymes having the whole of the above-identified amino acid sequences, the present invention includes another types of enzymes which comprise a part of any one of the amino acid sequences selected therefrom or which have both the action as the present non-reducing saccharide-forming enzyme and the above-identified optimum temperature. Examples of the amino acid sequences of such enzymes are those which contain, within the amino acid sequences, a partial amino acid sequence or an amino acid residue that are related to the expression of the properties of the present non-reducing saccharide-forming enzyme, and which one or more amino acids are replaced with different amino acids, added thereunto and/or deleted therefrom other than the above partial amino acid sequence or the amino acid residue. Examples of the amino acid sequences replaced with different amino acids as referred to in the present invention include those which less than 30% and preferably less than 20% of the amino acid sequences composing the amino acid sequence of SEQ ID NO:1 are replaced with another amino acids which have similar properties and structures to respective ones to be replaced. Examples of groups of such amino acids are a group of aspartic acid and

glutamic acid as acid amino acids, one of lysine, arginine, and histidine as basic amino acids, one of asparagine and glutamine as amid-type amino acids, one of serine and threonine as hydroxyamino acids, and one of valine, leucine and isoleucine as branched-chain amino acids. Examples of another amino acid sequences of the present enzyme containing a part of any one of the amino acid sequences selected from SEQ ID NOs:1 to 6 are those which might have a substantially similar stereo-structure to the one of the amino acid sequence of SEQ ID NO:1, i.e., replacement, deletion and/or addition of amino acid(s) are introduced into the amino acid sequence of SEQ ID NO:1. The stereo-structure of proteins is estimable by screening commercially available databases for stereo-structures of proteins which have amino acid sequences related to the aiming ones and have revealed stereo-structures, referencing the screened stereo-structures, and using commercially available soft wares for visualizing stereo-structures. The above-identified amino acid sequence of the present non-reducing saccharide-forming enzyme has a homology of at least 57%, preferably at least 70%, and more preferably at least 80% to SEQ ID NO:1.

As described above, the non-reducing saccharide-forming enzyme should not be restricted to a specific origin/source. Examples of such are those derived from microorganisms, i.e., those of the genus *Arthrobacter*, *Arthrobacter* sp. S34, FERM BP-6450, and its mutants. The mutants can be obtained by

treating in a usual manner *Arthrobacter* sp. S34, FERM BP-6450, with known mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine, ethyl methanesulfonate, ultraviolet, and transposon; screening the desired mutants capable of producing a non-reducing saccharide-forming enzyme and having an optimum temperature at temperatures in a medium temperature range, and usually at temperatures in the range of over 40°C but below 60°C. The enzyme from *Arthrobacter* sp. S34, FERM BP-6450, usually has the amino acid sequences of SEQ ID NOs:1 to 6. Another non-reducing saccharide-forming enzymes from microorganisms of mutants *Arthrobacter* sp. S34, FERM BP-6450, and another microorganisms comprise the whole or a part of any one of the amino acid sequences of SEQ ID NOs:1 to 6. Concrete examples of another enzymes include recombinant enzymes which act as the present non-reducing saccharide-forming enzyme and have an optimum temperature at temperatures in a medium temperature range, and usually at temperatures of over 40°C but below 60°C. The recombinant enzymes can be obtainable by applying the recombinant DNA technology for the DNA encoding the present non-reducing saccharide-forming enzyme, and have the whole or a part of any one of the amino acid sequences of SEQ ID NOs:1 to 6.

Most of the non-reducing saccharide-forming enzyme according to the present invention has the following physicochemical properties:

(1) Action

Forming a non-reducing saccharide having a trehalose structure as an end unit from a reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher;

(2) Molecular weight

About 75,000±10,000 daltons on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE);

(3) Isoelectric point (pI)

About 4.5±0.5 on isoelectrophoresis using ampholyte;

(4) Optimum temperature

About 50°C when incubated at pH 6.0 for 60 min;

(5) Optimum pH

About 6.0 when incubated at 50°C for 60 min;

(6) Thermal stability

Stable up to a temperature of about 55°C when incubated at pH 7.0 for 60 min; and

(7) pH Stability

Stable at pHs of about 5.0 to about 10.0 when incubated at 4°C for 24 hours.

The present non-reducing saccharide-forming enzyme can be obtained in a prescribed amount by the later described present process for producing the same.

The present invention provides a DNA encoding the present non-reducing saccharide-forming enzyme. Such a DNA is quite useful in producing the enzyme in the form of a recombinant protein. In general, the DNA includes those which encode the enzyme independently of its origin/source. Examples of such a DNA are those which contain the whole or a part of the nucleotide sequence of SEQ ID NO:7 or complementary ones thereunto. The DNA comprising the whole of the nucleotide sequence of SEQ ID NO:7 encodes the amino acid sequence of SEQ ID NO:1. The DNAs, which contain the whole or a part of the nucleotide sequence of SEQ ID NO:7, include those which have an amino acid sequence relating to the expression of the properties of the present non-reducing saccharide-forming enzyme, and have a nucleotide sequence corresponding to the animo acid sequence, and the nucleotide sequence of SEQ ID NO:7 introduced with a replacement, deletion and/or addition of one or more bases while retaining the nucleotide sequence relating to the expression of the properties of the present non-reducing saccharide-forming enzyme. The DNAs according to the present invention should include those which one or more bases are replaced with different ones based on the degeneracy of genetic code. Also the DNAs according to the present invention include those which comprise the nucleotide sequences that encode the present non-reducing saccharide-forming enzyme and further comprise additional one or more another nucleotide sequences selected from the group consisting of ribosome-binding sequences such as an initiation codon, termination codon, and Shine-Dalgarno

sequence; nucleotide sequences encoding signal peptides, recognition sequences for appropriate restriction enzymes; nucleotide sequences to regulate the expression of genes for promotor and enhancers; and terminators, all of which are generally used in recombinant DNA technology for producing recombinant proteins. For example, since a part of and the whole of the nucleotide sequence of SEQ ID NO:8 function as ribosome-binding sequences, DNAs to which the part of and the whole of the nucleotide sequence of SEQ ID NO:8 are ligated upstream of the nucleotide sequences encoding the present non-reducing saccharide-forming enzyme can be arbitrarily used in producing the enzyme as a recombinant protein.

As described above, the DNAs encoding the present non-reducing saccharide-forming enzyme should not be restricted to their origins/sources, and they are preparable by screening DNAs from different sources based on hybridization with a DNA comprising a nucleotide sequence which encodes at least a part of the amino acid sequence of the enzyme, eg., the amino acid sequence of SEQ ID NO:1. Actual examples of these sources are microorganisms of the genus *Arthrobacter*, and preferably, *Arthrobacter* sp. S34, FERM BP-6450, and its mutants, all of which produce the non-reducing saccharide-forming enzyme. To screen the microorganisms, conventional methods used in this field for screening or cloning DNAs such as screening methods of recombinant libraries, PCR method, and their modified methods. As a result of screening, the desired DNAs can be

obtained by collecting in a usual manner DNAs confirmed with the expected hybridization. Generally, the DNAs thus obtained comprise a part of or the whole of the nucleotide sequence of SEQ ID NO:7. For example, a DNA which comprises the whole of the nucleotide sequence of SEQ ID NO:7 is generally obtained from *Arthrobacter* sp. S34, FERM BP-6450. DNAs comprising a part of the nucleotide sequence of SEQ ID NO:7 can be obtained by similarly screening DNAs from microorganisms as sources other than the above strain, capable of producing the present non-reducing saccharide-forming enzyme. Such DNAs can be prepared by selecting DNAs, which encode the enzymes having the properties of the present enzyme, from DNAs into which have been introduced a replacement, addition and/or deletion of one or more bases of the above-mentioned DNAs by using one or more conventional mutation-introducing methods. The DNAs can be also obtained by applying conventional chemical syntheses based on the nucleotide sequence encoding the present non-reducing saccharide-forming enzyme, e.g., one of SEQ ID NO:7. Once in hand, the DNAs according to the present invention can be easily amplified to the desired level by applying or using PCR method and autonomously-replicable vectors.

The present DNA encoding the non-reducing saccharide-forming enzyme include those in the form of recombinant DNAs which the DNAs have been introduced into appropriate vectors. The recombinant DNAs can be relatively-easily preparable by recombinant DNA technology in general if only the DNAs are available. Any types of vectors can be used in the present

invention as long as they autonomously replicable in appropriate hosts. Examples of such vectors are pUC18, pBluescript II SK(+), pKK223-3, λgt•λC, etc., which use *Escherichia coli* as a host; pUB110, pTZ4, pC194, ρ11, ϕ1, ϕ105, etc., which use microorganisms of the genus *Bacillus*; and pHY300PLK, pHV14, TRp7, YEp7, pBS7, etc., which use two or more microorganisms as hosts. The methods to insert the present DNA into such vectors in the present invention may be conventional ones generally used in this field. A gene containing the present DNA and an autonomously-replicable vector are first digested with a restriction enzyme and/or ultrasonic disintegrator, then the resultant DNA fragments and vector fragments are ligated. The ligation is facilitated by the use of restriction enzymes which specifically act on the cleavage of the DNA, especially, *Kpn*I, *Acc*I, *Bam*HI, *Bst*XI, *Eco*RI, *Hind*III, *Not*I, *Pst*I, *Sac*I, *Sal*I, *Sma*I, *Spe*I, *Xba*I, *Xho*I, etc. To ligate DNA fragments and vectors, firstly they may be annealed if necessary, then subjected to the action of a DNA ligase *in vivo* or *in vitro*. The recombinant DNA thus obtained can be replicable without substantial limitation in an appropriate host.

The present DNA encoding the non-reducing saccharide-forming enzyme further includes transformants which the DNA has been introduced into appropriate vectors. The transformants can be easily preparable by introducing the DNA or recombinant DNA obtained in the above into appropriate hosts to transform them. As the hosts, microorganisms and cells from plants and animals,

which are used conventionally in this field and chosen depending on the vectors in the recombinant DNA, can be used. The microorganisms as hosts include those of the genera *Escherichia*, *Bacillus*, and *Arthrobacter*, and another actinomycetes, yeasts, fungi, etc. To introduce the present DNA into these host microorganisms, conventional competent cell method and protoplast method can be used. The present DNA, which encodes the non-reducing saccharide-forming enzyme introduced into the transformants in the present invention, may be present in a separatory form from chromosomes or in an incorporated form into chromosomes. The DNA incorporated into hosts' chromosomes has a character of being stably retained therein and may be advantageously used in producing the present recombinant protein.

The present non-reducing saccharide-forming enzyme can be obtained in a desired amount by a process for producing the enzyme characterized in that it comprises the steps of culturing microorganisms capable of producing the enzyme, and collecting the produced enzyme from the culture. The microorganisms used in the process can be used independently of the genus or the species as long as they produce the enzyme. Examples of such microorganisms are microorganisms of the genus *Arthrobacter*, *Arthrobacter* sp. S34, FERM BP-6450, and mutants thereof, as well as transformants obtainable by introducing the present DNA encoding the enzyme into appropriate hosts.

Any nutrient culture media used in culturing the

process for producing the present non-reducing saccharide-forming enzyme can be used as long as the aforesaid microorganisms grow therein and produce the enzyme without restriction to a specific nutrient culture medium. Generally, the nutrient culture media contain carbon and nitrogen sources, and if necessary minerals may be added. Examples of the carbon sources are saccharides such as dextrins, starches, partial starch hydrolysates, glucose, etc., and are saccharide-containing substances such as molasses and yeast extracts, and organic acids such as glucuronic acid and succinic acid. The concentration of the carbon sources is chosen depending on the types used, usually 30 w/v %, and preferably 15 w/w % or lower. Examples of the nitrogen sources appropriately used in the present invention are inorganic-nitrogen-containing substances such as ammonium salts, nitrate, etc.; organic-nitrogen-containing substances such as urea, corn steep liquor, casein, peptone, yeast extract, beef extract, etc. Depending on use, it is selectively used among inorganic ingredients such as salts of calcium, magnesium, potassium, sodium, phosphoric acid, manganese, zinc, iron, copper, molybdenum, cobalt, etc.

The culture conditions used for producing the present enzyme can be used selectively from appropriate conditions suitable for growing respective microorganisms used. For example, in the case of using microorganisms of the genus *Arthrobacter* including *Arthrobacter* sp. S34, FERM BP-6450, the cultivation temperature is usually in the range of 20-50°C, and preferably 25-37°C; the cultivation pH is usually in the range

of pH 4-10, and preferably pH 5-9; and the cultivation time is in the range of 10-150 hours. With these conditions, the microorganisms are cultured under aerobic conditions. When used transformants prepared by introducing into appropriate hosts the present DNA encoding the present non-reducing saccharide-forming enzyme, the transformants are cultured under aerobic conditions at conditions selected from the culture conditions such as the culture temperatures of 20-65°C, the culture pH of 2-9, and the culture time of 1-6 days, although they vary depending on the genus, species, strains or types of microorganisms and vectors. The cultures thus obtained generally contain the present enzyme in cell fractions. In the case of culturing transformants obtained by using as hosts the microorganisms of the genus *Bacillus*, the resulting cultures may contain the present enzyme in supernatant fractions depending on vectors used to transform the hosts. The content of the present enzyme in the cultures thus obtained is usually 0.01-1,000 units per ml of the culture, though it varies depending on the genus, species or strains of the microorganisms and culture conditions used.

The present non-reducing saccharide-forming enzyme is collected from the resulting cultures. The collection method is not restricted; The present enzyme can be obtained by separating and collecting any one of fractions of cells and culture supernatants found with a major activity of the enzyme, and if necessary subjecting the collected fraction to an appropriate purification method to collect a purified fraction containing the enzyme. To separate the fractions of cells and

culture supernatants of the cultures, conventional solid-liquid separation methods such as centrifugation and filtration using precoat filters and plain- and hollow fiber- membranes can be arbitrarily used. The desired fractions are collected from the separated fractions of cells and culture supernatant. For the fraction of cells, the cells are disrupted into a cell disruptant which is then separated into a cell extract and an insoluble cell fraction, followed by collecting either of the desired fractions. The insoluble cell fraction can be solubilized by conventional methods, if necessary. As a method to disrupt cells, any one of techniques of ultrasonication, treatment with cell-wall-lysing enzymes such as lysozyme and glucanase, and load of mechanical press can be arbitrarily used. To disrupt cells the cultures can be directly treated with any one of the above techniques, and then resulting mixtures are treated with any one of the above solid-liquid separation methods to collect a liquid fraction. Thus a cell extract can be arbitrarily obtained.

The methods used for more purifying the present non-reducing saccharide-forming enzyme include conventional ones to purify saccharide-related enzymes in general such as salting out, dialysis, filtration, concentration, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, reverse-phase chromatography, affinity chromatography, gel electrophoresis and, isoelectric point electrophoresis. These methods can be used in combination depending on purposes. From the resulting fractions separated

by these methods, fractions with a desired activity assayed by the method for non-reducing saccharide-forming enzyme are collected to obtain the present non-reducing saccharide-forming enzyme purified to a desired level. According to the methods in the later described Examples, the present enzyme can be purified up to an electrophoretically homogenous level. As described above, the present method provide the present non-reducing saccharide-forming enzyme in the form of a culture, cell fraction, fraction of culture supernatant, cell disruptant, cell extract, soluble and insoluble cell-fraction, partially purified enzyme fraction, and purified enzyme fraction. These fractions may contain another type of the present trehalose-releasing enzyme. The non-reducing saccharide-forming enzyme thus obtained can be immobilized in a usual manner before use. The methods for immobilization are, for example, binding method to ion exchangers, covalent bonding/adsorption to and on resins and membranes, and entrapping immobilization method using high molecular weight substances. The non-reducing saccharide-forming enzyme thus obtained can be arbitrarily used in processes for producing saccharides including the later described present process for producing saccharide. Particularly, since the present non-reducing saccharide-forming enzyme has an optimum temperature in a medium temperature range and preferably has an optimum pH in an acid pH range, it can be advantageously used to produce saccharides when used in combination with the later described present trehalose-releasing enzyme, starch-debranching enzyme having an optimum pH in an

acid pH range, and cyclomaltodextrin glucanotransferase that effectively acts at medium temperature range.

Explaining the present trehalose-releasing enzyme based on the amino acid sequence, the enzyme has the amino acid sequence of SEQ ID NO:9 as a whole, and has the amino acid sequences of SEQ ID NOs:10 to 16 as partial amino acid sequences in some cases. In addition to these enzymes having the whole of the above-identified amino acid sequences, the present invention includes another types of enzymes which comprise a part of any one of the amino acid sequences selected therefrom or which have both the action as the present trehalose-releasing enzyme and the above-identified optimum temperature. Examples of the amino acid sequences of such enzymes are those which contain, within the amino acid sequences, a partial amino acid sequence or an amino acid residue which relate to the expression of the properties of the present non-reducing saccharide-forming enzyme, and which one or more amino acids are replaced with different amino acids, added thereunto and/or deleted therefrom other than the above partial amino acid sequence or the amino acid residue. Examples of amino acid sequences replaced with different amino acids as referred to in the present invention include those which less than 30% and preferably less than 20% of the amino acid sequences composing the amino acid sequence of SEQ ID NO:9 are replaced with another amino acids which have similar properties and structures to respective ones to be replaced. Examples of groups of such amino acids are a group of aspartic acid and glutamic acid as acid amino acids, one of

lysine, arginine, and histidine as basic amino acids, one of asparagine and glutamine as amid-type amino acids, one of serine and threonine as hydroxyamino acids, and one of valine, leucine and isoleucine as branched-chain amino acids. Examples of another amino acid sequences of the enzyme containing a part of any one of the amino acid sequences selected from SEQ ID NOs:9 to 16 are those which might have a substantially similar stereo-structure to the one of the amino acid sequence of SEQ ID NO:9, i.e., replacement, deletion and/or addition of amino acid(s) are introduced into the amino acid sequence of SEQ ID NO:9. The stereo-structure of proteins is estimable by screening commercially available databases for stereo-structures of proteins which have amino acid sequences related to the aiming ones and have revealed stereo-structures, referencing the screened stereo-structures, and using commercially available soft wares for visualizing stereo-structures. The above-identified amino acid sequence of the present trehalose-releasing enzyme has a homology of at least 60%, preferably at least 70%, and more preferably at least 80% to SEQ ID NO:9.

As described above, the trehalose-releasing enzyme should not be restricted to a specific origin/source. Examples of such are those derived from microorganisms, i.e., those of the genus *Arthrobacter*, *Arthrobacter* sp. S34, FERM BP-6450, and mutants thereof. The mutants can be obtained by treating in a usual manner *Arthrobacter* sp. S34, FERM BP-6450, with known mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine, ethyl

methanesulfonate, ultraviolet, and transposon; screening the desired mutants capable of producing a non-reducing saccharide-forming enzyme and having an optimum temperature at temperatures in a medium temperature range, and usually at temperatures in the range of over 45°C but below 60°C. The enzyme from *Arthrobacter* sp. S34, FERM BP-6450, usually has the amino acid sequences of SEQ ID NOs:9 to 16. Another non-reducing saccharide-forming enzymes from microorganisms of mutants *Arthrobacter* sp. S34, FERM BP-6450, and another microorganisms comprise the whole or a part of any one of the amino acid sequences of SEQ ID NOs:9 to 16. Concrete examples of another enzymes include recombinant enzymes which act as the present trehalose-releasing enzyme and have an optimum temperature at temperatures in a medium temperature range, and usually at temperatures of over 45°C but below 60°C. The recombinant enzymes can be obtainable by applying the recombinant DNA technology for the DNA encoding the present trehalose-releasing enzyme, and have the whole or a part of any one of the amino acid sequences of SEQ ID NOs:9 to 16.

Most of the trehalose-releasing enzyme according to the present invention has the following physicochemical properties:

(1) Action

Specifically hydrolyses a non-reducing saccharide having a trehalose structure as an end unit at a site between a part of the

trehalose structure and a part of the resting;

(2) Molecular weight

About 62,000±5,000 daltons on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE);

(3) Isoelectric point (pI)

About 4.7±0.5 on isoelectrophoresis using ampholyte;

(4) Optimum temperature

About 50°C to about 55°C when incubated at pH 6.0 for 30 min;

(5) Optimum pH

About 6.0 when incubated at 50°C for 30 min;

(6) Thermal stability

Stable up to a temperature of about 50°C when incubated at pH 7.0 for 60 min; and

(7) pH Stability

Stable at pHs of about 4.5 to about 10.0 when incubated at 4°C for 24 hours.

The present trehalose-releasing enzyme can be obtained in a prescribed amount by the later described present process for producing the same.

The present invention provides a DNA encoding the present trehalose-releasing enzyme. Such a DNA is quite useful in producing the enzyme in the form of a recombinant protein. In general, the DNA includes those which encode the enzyme independently of its origin/source. Examples of such a DNA are

those which contain the whole or a part of the nucleotide sequence of SEQ ID NO:17 or complementary ones thereunto. The DNA comprising the whole of the nucleotide sequence of SEQ ID NO:17 encodes the amino acid sequence of SEQ ID NO:9. The DNAs, which contain the whole or a part of the nucleotide sequence of SEQ ID NO:17, include those which have a nucleotide sequence corresponding to an amino acid sequence relating to the expression of the properties of the present non-reducing saccharide-forming enzyme, and have the nucleotide sequence of SEQ ID NO:17 introduced with a replacement, deletion and/or addition of one or more bases while retaining the nucleotide sequence relating to the expression of the properties of the present trehalose-releasing enzyme. The DNAs according to the present invention should include those which one or more bases are replaced with different ones based on the degeneracy of genetic code. Also the DNAs according to the present invention include those which comprise the nucleotide sequences that encode the present trehalose-releasing enzyme and further comprise additional one or more another nucleotide sequences selected from the group consisting of ribosome-binding sequences such as an initiation codon, termination codon, and Shine-Dalgarno sequence; nucleotide sequences encoding signal peptides, recognition sequences for appropriate restriction enzymes; nucleotide sequences to regulate the expression of genes for promotor and enhancers; and terminators, all of which are generally used in recombinant DNA technology for producing recombinant proteins. For example, since a part of and the

whole of the nucleotide sequence of SEQ ID NO:8 function as ribosome-binding sequences, DNAs to which the part of and the whole of the nucleotide sequence of SEQ ID NO:8 are ligated upstream of the nucleotide sequences encoding the present trehalose-releasing enzyme can be arbitrarily used in producing the enzyme as a recombinant protein.

As described above, the DNAs encoding the present trehalose-releasing enzyme should not be restricted to their origins/sources, and they are preparable by screening DNAs from different sources based on hybridization with a DNA comprising a nucleotide sequence which encodes at least a part of the amino acid sequence of the enzyme, eg., the amino acid sequence of SEQ ID NO:9. Actual examples of these sources are microorganisms of the genus *Arthrobacter*, and preferably, *Arthrobacter* sp. S34, FERM BP-6450, and its mutants, all of which produce the non-reducing saccharide-forming enzyme. To screen the microorganisms, conventional methods used in this field for screening or cloning DNAs such as screening methods of recombinant libraries, PCR method, and their modified methods. As a result of screening, the desired DNAs can be obtained by collecting in a usual manner DNAs confirmed with the expected hybridization. Generally, the DNAs thus obtained comprise a part of or the whole of the nucleotide sequence of SEQ ID NO:17. For example, a DNA which comprises the whole of the nucleotide sequence of SEQ ID NO:17 is generally obtained from *Arthrobacter* sp. S34, FERM BP-6450. DNAs comprising a part of the nucleotide

sequence of SEQ ID NO:17 can be obtained by similarly screening DNAs from microorganisms as sources other than the above strain, capable of producing the trehalose-releasing enzyme. Such DNAs can be prepared by selecting DNAs, which encode the enzymes having the properties of the enzyme, from DNAs into which have been introduced a replacement, addition and/or deletion of one or more bases of the above-mentioned DNAs by using one or more conventional mutation-introducing methods. The DNAs can be also obtained by applying conventional chemical syntheses based on the nucleotide sequence encoding the present trehalose-releasing enzyme, e.g., one of SEQ ID NO:17. Once in hand, the DNAs according to the present invention can be easily amplified to the desired level by applying or using PCR method and autonomously-replicable vectors.

The present DNA encoding the trehalose-releasing enzyme include those in the form of recombinant DNAs which the DNAs have been introduced into appropriate vectors. The recombinant DNAs can be relatively-easily preparable by recombinant DNA technology in general if only the DNAs are available. Any types of vectors can be used in the present invention as long as they autonomously replicable in appropriate hosts. Examples of such vectors are pUC18, pBluescript II SK(+), pKK223-3, λgt•λC, etc., which use *Escherichia coli* as a host; pUB110, pTZ4, pC194, ρ11, ϕ1, ϕ105, etc., which use microorganisms of the genus *Bacillus;* and pHY300PLK, pHV14, TRp7, YEp7, pBS7, etc., which use two or more microorganisms as

hosts. The methods to insert the present DNA into such vectors in the present invention may be conventional ones generally used in this field. A gene containing the present DNA and an autonomously-replicable vector are first digested with a restriction enzyme and/or ultrasonic disintegrator, then the resultant DNA fragments and vector fragments are ligated. The ligation is facilitated by the use of restriction enzymes which specifically act on the cleavage of the DNA, especially, *Kpn*I, *Acc*I, *Bam*HI, *Bst*XI, *Eco*RI, *Hind*III, *Not*I, *Pst*I, *Sac*I, *Sal*I, *Sma*I, *Spe*I, *Xba*I, *Xho*I, etc. To ligate DNA fragments and vectors, firstly they may be annealed if necessary, then subjected to the action of a DNA ligase *in vivo* or *in vitro*. The recombinant DNA thus obtained can be replicable without substantial limitation in an appropriate host.

The present DNA encoding the trehalose-releasing enzyme further includes transformants which the DNA has been introduced into appropriate vectors. The transformants can be easily preparable by introducing the DNA or recombinant DNA obtained in the above into appropriate hosts to transform them. As the hosts, microorganisms and cells from plants and animals, which are used conventionally in this field and chosen depending on the vectors in the recombinant DNA, can be used. The microorganisms as hosts include those of the genera *Escherichia*, *Bacillus*, and *Arthrobacter*, and another actinomycetes, yeasts, fungi, etc. To introduce the present DNA into these host microorganisms, conventional competent cell method and

protoplast method can be used. The present DNA, which encodes the trehalose-releasing enzyme introduced into the transformants in the present invention, may be present in a separatory form from chromosomes or in an incorporated form into chromosomes. The DNA incorporated into hosts' chromosomes has a character of being stably retained therein and may be advantageously used in producing the present recombinant protein.

The aforesaid techniques used for obtaining the present DNAs including recombinant DNAs and transformants, and the techniques for obtaining the DNAs and recombinant proteins are commonly used in the art; For example, J. Sumbruck et al. in "*Molecular Cloning A Laboratory Manual*", 2nd edition, published by Cold Spring Harbor Laboratory Press (1989), discloses in detail methods for obtaining desired DNAs and applications for production use of the obtained DNAs. For example, Japanese Patent No. 2,576,970 discloses a method for stabilizing a transformed DNA, which uses as a host a microorganism defective in an aiming gene. Japanese Patent Kokai No. 157,987/88 discloses a vector which effectively expresses an aiming DNA in microorganisms of the genus *Bacillus*. Japanese Patent Kohyo No. 502,162/93 discloses a method for stably introducing a desired DNA into a bacterial chromosome. Japanese Patent Kohyo No. 506,731/96 discloses an efficient production method of a starch hydrolysing enzyme, using recombinant DNA technology. Japanese Patent Kohyo Nos. 500,543/97 and 500,024/98 disclose a host-vector system using

fungi for efficient production of recombinant proteins. These methods conventionally used in the art are arbitrarily applicable for the present invention.

In the art, when the desired DNAs are available by the above methods, there have been commonly provided transformants which the DNAs are introduced into appropriate plants and animals, i.e., transgenic plants and animals. The present DNA, which encodes the non-reducing saccharide-forming enzyme and the trehalose-releasing enzyme in the form of a DNA introduced into appropriate hosts, also includes the transgenic plants and animals. To obtain the transgenic animals, it is obtained as a whole by a process comprising the DNA which encodes either of the present enzymes alone or together with other desired DNA such as a promotor and enhancer into an appropriate vector selected depending on the species of the host animal, introducing the resulting recombinant DNA into a fertilized egg or embryonic stem cell from the host animal by a method such as micro-injection and electroporation, or by an infection method using recombinant viruses containing the recombinant DNA. Examples of the host animals are conventional experimental rodents such as mice, rats, and hamsters; and mammals conventionally used as domestic animals such as goats, sheep, pigs, and cows, all of which have an advantage of being bred easily. The resulting cells introduced with the DNA are transplanted in uterine tube or uterus of a pseudopregnancy female animal of the same species as the cells. Thereafter, transgenic animals, which have been introduced with the DNA

encoding the present enzymes by applying hybridization or PCR method, are obtained from newborns in a natural or cesarean sectional manner. Thus the present DNA in the form of a transgenic animal can be obtained. Referring to transgenic animals, they are disclosed in detail in "*Jikken-Igaku-Bessatsu-Shin-Idennshi-Kogaku-Handbook*" (Handbook of Genetic Engineering), pp. 269-283 (1996), edited by Masami MATSUMURA, Hiroto OKAYAMA, and Tadashi YAMAMOTO, published by Yodosha Co., Ltd., Tokyo, Japan. The method for obtaining transgenic plants comprises, for example, providing a plasmid as a vector of a microorganism of the genus *Agrobacterium* infectious to plants, introducing the DNA encoding either of the present enzymes into the vector, and either introducing the resulting recombinant DNA into plant bodies or protoplasts, or coating heavy metal particles with a DNA including nucleotide sequence encoding either of the present enzymes and directly injecting the coated particles into plant bodies or protoplasts using a particle gun. Although various types of plants can be used as host plants, they generally include edible plants such as potato, soybean, wheat, burley, rice, corn, tomato, lettuce, alfalfa, apple, peach, melon, etc. By applying hybridization or PCR method for the above transformed plant bodies and protoplasts, transformants containing the desired DNA are selected. The transformed protoplasts can be regenerated into plant bodies as the present DNA in the form of transgenic plants. The techniques of transgenic plants are generally disclosed in

Genetic Engineering, edited by Jane K. Setlow, published by Plenum Publishing Corporation, NY, USA, Vol. 16, pp. 93-113 (1994). The DNA in the form of the aforesaid transgenic animals and plants can be used as sources of the present non-reducing saccharide-forming enzyme and/or trehalose-releasing enzyme, and used as edible plants and animals which contain trehalose or non-reducing saccharide having a trehalose structure.

The present trehalose-releasing enzyme can be obtained in a desired amount by the present process for producing the enzyme which is characterized in that it comprises culturing a microorganism capable of producing the enzyme in a nutrient culture medium, and collecting the produced enzyme from the resulting culture. Any microorganisms can be used in the present process independently of their genus and species as long as they produce the present trehalose-releasing enzyme. Examples of such microorganisms are those of the genus *Arthrobacter*, *Arthrobacter* sp. S34, FERM BP-6450, and mutants thereof, as well as transformants obtainable by introducing the present DNA encoding the enzyme into appropriate host microorganisms.

Any nutrient culture media for culturing the process for producing the present trehalose-releasing enzyme can be used as long as the aforesaid microorganisms grow therein and produce the enzyme without restriction to a specific nutrient culture medium. Generally, the nutrient culture media contain carbon and nitrogen sources, and if necessary minerals may be added.

Examples of the carbon sources are saccharides such as dextrins, starches, partial starch hydrolysates, glucose, etc., and are saccharide-containing substances such as molasses and yeast extracts, and organic acids such as glucuronic acid and succinic acid. The concentration of the carbon sources is chosen depending on the types used, usually 30 w/v %, and preferably 15 w/w % or lower. Examples of the nitrogen sources appropriately used in the present invention are inorganic-nitrogen-containing substances such as ammonium salts, nitrate, etc.; organic-nitrogen-containing substances such as urea, corn steep liquor, casein, peptone, yeast extract, beef extract, etc. Depending on use, it is selectively used among inorganic ingredients such as salts of calcium, magnesium, potassium, sodium, phosphoric acid, manganese, zinc, iron, copper, molybdenum, cobalt, etc.

The culture conditions used for producing the present trehalose-releasing enzyme can be used selectively from appropriate conditions suitable for growing respective microorganisms used. For example, in the case of using microorganisms of the genus *Arthrobacter* including *Arthrobacter* sp. S34, FERM BP-6450, the cultivation temperature is usually in the range of 20-50°C, and preferably 25-37°C; the cultivation pH is usually in the range of pH 4-10, and preferably pH 5-9; and the cultivation time is in the range of 10-150 hours. With these conditions, the microorganisms are cultured under aerobic conditions. When used transformants prepared by introducing into appropriate hosts the present DNA encoding the trehalose-

releasing enzyme, the transformants are cultured under aerobic conditions at conditions selected from the culture conditions such as the culture temperatures of 20-65°C, the culture pH of 2-9, and the culture time of 1-6 days, although they vary depending on the genus, species, strains or types of microorganisms and vectors. The cultures thus obtained generally contain the enzyme in cell fractions. In the case of culturing transformants obtained by using as hosts the microorganisms of the genus *Bacillus*, the resulting cultures may contain the enzyme in supernatant fractions depending on vectors used to transform the hosts. The content of the enzyme in the cultures thus obtained is usually 0.01-3,000 units per ml of the culture, though it varies depending on the genus, species or strains of the microorganisms and culture conditions used.

The present trehalose-releasing enzyme is collected from the resulting cultures. The collection method is not restricted; The enzyme can be obtained by separating and collecting any one of fractions of cells and culture supernatants found with a major activity of the enzyme, and if necessary subjecting the collected fraction to an appropriate purification method to collect a purified fraction containing the enzyme. To separate the fractions of cells and culture supernatants of the cultures, conventional solid-liquid separation methods such as centrifugation and filtration using precoat filters and plain- and hollow fiber- membranes can be arbitrarily used. The desired fractions are collected from the separated fractions of cells and culture supernatant. For the

fraction of cells, the cells are disrupted into a cell disruptant which is then separated into a cell extract and an insoluble cell fraction, followed by collecting either of the desired fractions. The insoluble cell fraction can be solubilized by conventional methods, if necessary. As a method to disrupt cells, any one of techniques of ultrasonication, treatment with cell-wall-lysing enzymes such as lysozyme and glucanase, and load of mechanical press can be arbitrarily used. To disrupt cells the cultures can be directly treated with any one of the above techniques, and then resulting mixtures are treated with any one of the above solid-liquid separation methods to collect a liquid fraction. Thus a cell extract can be arbitrarily obtained.

The methods used for more purifying the present trehalose-releasing enzyme include conventional ones to purify saccharide-related enzymes in general such as salting out, dialysis, filtration, concentration, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, reverse-phase chromatography, affinity chromatography, gel electrophoresis and, isoelectric point electrophoresis. These methods can be used in combination depending on purposes. From the resulting fractions separated by these methods, fractions with a desired activity assayed by the method for trehalose-releasing enzyme are collected to obtain the enzyme purified to a desired level. According to the methods in the later described Examples, the present enzyme can be purified up to an electrophoretically homogenous level. As

described above, the present method provide the present trehalose-releasing enzyme in the form of a culture, cell fraction, fraction of culture supernatant, cell disruptant, cell extract, soluble and insoluble cell-fraction, partially purified enzyme fraction, and purified enzyme fraction. These fractions may contain another type of the present non-reducing saccharide-forming enzyme. The present trehalose-releasing enzyme thus obtained can be immobilized in a usual manner before use. The methods for immobilization are, for example, binding method to ion exchangers, covalent bonding/adsorption to and on resins and membranes, and entrapping immobilization method using high molecular weight substances. The trehalose-releasing enzyme thus obtained can be arbitrarily used in processes for producing saccharides including the later described present process for producing saccharide. Particularly, since the trehalose-releasing enzyme has an optimum temperature in a medium temperature range and preferably has an optimum pH in an acid pH range, it can be advantageously used to produce saccharides when used in combination with the later described present trehalose-releasing enzyme, starch-debranching enzyme having an optimum pH in an acid pH range, and cyclomatodextrin glucanotransferase that effectively acts at temperatures in a medium temperature range.

The present invention provides a process for producing saccharides comprising non-reducing saccharides by using the aforesaid present enzymes; the process comprising the steps of allowing the non-reducing saccharide-forming enzyme and/or the

trehalose-releasing enzyme to act on reducing partial starch hydrolysates to form non-reducing saccharides, and collecting the resulting non-reducing saccharides or saccharide compositions with a lesser reducibility. In the process, the use of one or more another types of non-reducing saccharide-forming enzymes and trehalose-releasing enzymes other than the present enzymes, and other saccharide-related enzymes should not be excluded from the present invention. The reducing partial starch hydrolysates used in the process can be used independently of their origins/sources. The non-reducing saccharides as referred to in the present invention include non-reducing saccharides in general such as trehalose and those having a trehalose structure.

The reducing partial starch hydrolysates used in the present process for producing saccharides can be obtained, for example, by liquefying starches or amylaceous substances by conventional methods. The starches include terrestrial starches such as corn starch, rice starch, and wheat starch; and subterranean starches such as potato starch, sweet potato starch, and tapioca starch. To liquefy these starches, they are generally suspended in water into starch suspensions, preferably, those with a concentration of at least 10 w/w %, and more preferably those with a concentration of about 20 to about 50 w/w %, and treated with mechanical, acid and/or enzymatic treatments. Relatively-lower degree of liquefaction is satisfactorily used, preferably, DE (dextrose equivalent) of less than 15, and more preferably DE of less than 10. When

liquefied with acids, the starches are treated with hydrochloric acid, phosphoric acid, oxalic acid, etc., and then the resulting mixtures are neutralized with calcium carbonate, calcium oxide, sodium carbonate, etc., to desired pHs before use. To liquefy the starches with enzymes, α-amylase, particularly, and thermostable liquefying α-amylase are satisfactorily used. The liquefied starches thus obtained can be further subjected to the action of α-amylase, maltotriose-forming amylase, maltotetraose-forming amylase, maltopentaose-forming amylase, maltohexaose-forming amylase, etc., and the resulting reaction mixtures can be used as the reducing partial starch hydrolysates. The properties of the starch-related enzymes are described in detail in *Handbook of Amylases and Related Enzymes*, pp.18-81, and pp. 125-142 (1988), published by Pergamon Press.

The reducing partial starch hydrolysates thus obtained are subjected to the action of the present non-reducing saccharide-forming enzyme and/or trehalose-releasing enzyme, and if necessary further subjected to the action of one or more starch-related enzymes such as α-amylase, β-amylase, glucoamylase, starch debranching enzymes such as isoamylase and pullulanase, cyclomaltodextrin glucanotransferase, α-glucosidase, and β-fructofuranosidase. Conditions used for enzymatic reactions are those suitable for enzymes used; Usually they are selected from pHs 4-10 and temperatures of 20-70°C , and preferably pHs 5-7 and temperatures of 30-60°C. Particularly, non-reducing saccharides can be effectively produced by enzymatic reactions at temperatures in a medium

temperature range, i.e., temperatures of over 40°C but below 60°C or over 45°C but below 60°C, and pHs of slight acid or acid pH conditions. The order of allowing the enzymes to act on reducing partial starch hydrolysates is not restricted; one proceeds or follows another one, or plural enzymes can be arbitrarily allowed to act on substrates simultaneously.

The amount of enzymes is appropriately set depending on enzymatic conditions and reaction times, and final uses of non-reducing saccharides or less-reducible saccharide compositions containing thereof. For the present non-reducing saccharide-forming enzyme and trehalose-releasing enzyme, the former is used in an amount of about 0.01 to about 100 units/g solid of reducing partial starch hydrolysates, and the latter is used in an amount of about 1 to about 10,000 units/g solid of reducing partial starch hydrolysates. Cyclomatodextrin glucanotransferase is used in an amount of about 0.05 to about 500 units/g reducing partial starch hydrolysates, d.s.b. The reaction mixtures obtained with these enzymes usually contain trehalose, α-glucosyltrehalose, α-maltosyltrehalose, α-maltotriosyltrehalose, α-maltotetraosyltrehalose, or α-maltopentaosyltrehalose. In the above process, when used in combination, the present non-reducing saccharide-forming enzyme and trehalose-releasing enzyme along with a starch debranching enzyme and cyclomatodextrin glucanotransferase characteristically more produce a large amount of trehalose and a relatively-lower molecular weight of non-reducing saccharide having a trehalose structure.

From the resulting reaction mixtures, non-reducing saccharides and saccharide compositions with a lesser reducibility are collected. In these production steps, conventionally used processed for saccharides can be appropriately selected. The resulting reaction mixtures are subjected to filtration and centrifugation to remove insoluble substances, and then the resultant solutions are purified by decoloration with an activated charcoal, desalted with ion exchangers in H- and OH-form, and concentrated into syrupy products. If necessary, the syrupy products can be further purified into non-reducing saccharides with a relatively-high purity; In the purification, one or more methods, for example, column chromatographic fractionations such as ion-exchange column chromatography, column chromatography using an activated charcoal or a silica gel; separatory sedimentation using organic acids such as acetone and alcohol; separation using membranes with an appropriate separability; and alkaline treatments to decompose and remove the remaining reducing saccharides. In particular, ion-exchange column chromatography can be suitably used in the present invention as an industrial-scale preparation of the object saccharides. Non-reducing saccharides with an improved purity can be arbitrary prepared by, for example, column chromatography using a strongly-acid cation exchange resin as described in Japanese Patent Kokai Nos. 23,799/83 and 72,598/83 to remove concomitant saccharides. In this case, any of fixed-bed, moving bed, and semi-moving methods can be employed.

If necessary, the resulting non-reducing saccharides or a relatively-low reducing saccharides containing the non-reducing saccharides can be hydrolyzed by amylases such as α-amylase, β-amylase, glucoamylase and α-glucosidase to control their sweetness and reducing power or to lower their viscosity; and the products thus obtained can be further treated with processings where the remaining reducing saccharides are hydrogenated into sugar alcohols to diminish their reducing powder. Particularly, trehalose can be easily prepared by allowing glucoamylase or α-glucosidase to act on the non-reducing saccharides or relatively-low reducing saccharides containing the non-reducing saccharides. A high trehalose content fraction is obtainable by allowing glucoamylase or α-glucosidase to act on these saccharides to form a mixture of trehalose and glucose, and subjecting the mixture to the aforesaid purification methods such as column chromatography using ion exchangers to remove glucose. The high trehalose content fraction can be arbitrary purified and concentrated into a syrupy product. If necessary, the syrupy product can be concentrated into a supersaturated solution, followed by crystallizing hydrous or anhydrous crystalline trehalose and recovering the resultant crystal.

To produce hydrous crystalline trehalose, an about 65-90 w/w % solution of trehalose with a purity of about 60 w/w % or higher is placed in a crystallizer, and if necessary in the presence of 0.1-20 w/v % seed crystal, gradually cooled while stirring at a temperature of 95°C or lower, and preferably at

a temperature of 10-90°C to obtain a massecuite containing hydrous crystalline trehalose. Continuous crystallization method to effect crystallization under concentrating conditions *in vacuo* can be arbitrarily used.

Conventional methods such as separation, block pulverization, fluidized-bed granulation, and spray drying can be employed in the invention to prepare from the massecuite hydrous crystalline trehalose or crystalline saccharides containing the trehalose crystal.

In the case of separation, massecuites are usually subjected to a basket-type centrifuge to separate hydrous crystalline trehalose from a mother liquor, and if necessary the hydrous crystalline trehalose is washed by spraying with a small amount of cold water to facilitate the preparation of hydrous crystalline trehalose with a higher purity. In the case of spray drying, crystalline saccharides with no or substantially free of hygroscopicity are easily prepared by spraying massecuites with a concentration of 70-85 w/w %, on a dry solid basis (d.s.b.), and a crystallinity of about 20-60%, d.s.b., from a nozzle by a high-pressure pump; drying the resultant products with air heated to 60-100°C which does not melt the resultant crystalline powders; and aging the resultant powders for about 1 to about 20 hours while blowing thereto air heated to 30-60°C. In the case of block pulverization, crystalline saccharides with no or substantially free of hygroscopicity are easily prepared by allowing massecuites with a moisture content of 10-20 w/w % and a crystallinity of about 10-60%, d.s.b., to

stand for about 0.1 to about 3 days to crystallize and solidify the whole contents into blocks; and pulverizing or cutting the resultant blocks.

To produce anhydrous crystalline trehalose, the hydrous crystalline trehalose obtained in the above is dried at a normal or reduced pressure at temperatures of 70-160°C, and preferably at 80-100°C; or a relatively-high concentration and content trehalose solution with a moisture content of less than 10% is placed in a crystallizer, stirred in the presence of a seed crystal at temperatures of 50-160°C, and preferably 80-140°C to produce a massecuite containing anhydrous crystalline trehalose, and treating the massecuite with methods such as block pulverization, fluidized-bed granulation, and spray drying under relatively-high temperatures and drying conditions.

The non-reducing saccharides or saccharide composition, containing thereof with a relatively-low reducibility, thus obtained are low in reducibility and satisfactory in stability; they do not become browning, form indisagreeable smell, and deteriorate the following another materials when mixed and processed with another materials, for example, amino-acid-containing substances such as amino acids, oligopeptides, and proteins. Even with a relatively-low reducibility, the above-identified saccharides have a relatively-low viscosity, and those with a relatively-low average glucose polymerization degree have a relatively-high quality and sweetness. These saccharides can be arbitrarily used in the fields of foods, cosmetics, and pharmaceuticals,

etc., as disclosed in Japanese Patent Kokai Nos.66,187/96, 66,188/96, 73,482/96, 73,506/96, 73,504/96, 336,363/96, 9,986/97, 154,493/97, 252,719/97, 66,540/98, and 168,093/98; and Japanese Patent Application Nos. 236,441/97, 256,219/97, 268,202/97, 274,962/97, 320,519/97, 338,294/97, 55,710/98, 67,628/98, 134,553/98 and 214,375/98, which were all applied for by the same applicant as the present applicant.

The following examples describe the present invention in more detail:

Example 1

Microorganism capable of producing non-reducing saccharide-forming enzyme and trehalose-releasing enzyme

The present inventors widely screened soils to isolate a microorganism capable of producing non-reducing saccharide-forming enzyme and trehalose-releasing enzyme. As a result, they isolated a microorganism with such a property from a soil in Ako, Hyogo, Japan, and identified the microorganisms in accordance with the method as described in "*Biseibutsu-no-Bunrui-to-Dotei*" (Classification and Identification of Microorganisms), edited by Takeji Hasegawa, published by Japan Scientific Societies Press, Tokyo, Japan (1985). The results were as follows:

Results on cell morphology

(1) Characteristics of cells when incubated at 37°C in nutrient agar broth

Usually existing a rod form of 0.4-0.5 x 0.8-1.2 μm; Existing in a single form but uncommonly

existing in a polymorphic form;

Free of motility;

Asporogenic;

Non-acid fast; and

Gram stain : Positive.

(2) Characteristics of cells when incubated at 37°C in EYG nutrient agar

Exhibiting a growth cycle of rods and cocci.

Results on Cultural property

(1) Characteristics of colony formed when incubated at 37°C in nutrient agar broth plate

Shape : Circular colony having a diameter of about 1-2 mm after 2-days incubation;

Rim : Entire;

Projection : Convex;

Gloss : Moistened gloss;

Surface : Plain; and

Color : Semi-transparent or cream.

(2) Characteristics of colony formed when incubated at 37°C in nutrient agar broth slant

Growth : Satisfactory; and

Shape : Thread-like.

(3) Characteristics of colony formed when incubated at 37°C in agar slant with yeast

extract and peptone

Growth : Satisfactory; and

Shape : Thread-like.

(4) Characteristics of colony formed when stab-cultured at 27°C in nutrient gelatin broth

Not liquefying gelatin.

Results on physiological properties

(1) Methyl red test : Negative

(2) VP-test : Positive

(3) Formation of indole : Negative

(4) Formation of hydrogen sulfide : Negative

(5) Hydrolysis of starch : Positive

(6) Liquefaction of gelatin : Negative

(7) Utilization of citric acid : Positive

(8) Utilization of inorganic nitrogen source:

Utilizing nitrate but not ammonium salts

(9) Formation of pigment : Non

(10) Urease : Negative

(11) Oxidase : Negative

(12) Catalase : Positive

(13) Growth range : Growing at pHs of 4.5-8.0 and temperatures of 20-50°C; and

Optimum temperatures of 30-45°C.

(14) Oxygen requirements : Aerobic

(15) Utilization of carbon sources

L-Arabinose : Assimilated

D-Glucose : Assimilated

D-Fructose : Not assimilated

D-Galactose : Not assimilated

L-Rhamnose : Not assimilated

D-Xylose : Not assimilated

D-Mannose : Assimilated

Raffinose : Not assimilated

Trehalose : Not assimilated

Sucrose : Not assimilated

Maltose : Not assimilated

Lactose : Not assimilated

D-Dulcitol : Not assimilated

D-Mannitol : Not assimilated

Gluconic acid : Assimilated

Succinic acid : Assimilated

Nicotinic acid : Not assimilated

L-Maleic acid : Assimilated

Acetic acid : Assimilated

Lactic acid : Assimilated

(16) Acid formation from sugars

L-Arabinose : Slightly formed

D-Glucose : Slightly formed

D-fructose : Not formed

D-Galactose : Slightly formed

L-Rhamnose : Slightly formed

D-Xylose : Slightly formed

Glycerol : Slightly formed

Raffinose : Not formed

Trehalose : Slightly formed

Sucrose : Slightly formed

Maltose : Slightly formed

Lactose : Not formed

(17) Utilization of amino acid

Not utilizing sodium L-glutamate, sodium L-aspartate, L-histidine and L-arginine.

(18) Decarboxylase test on amino acid

Negative against L-lysine, L-ornithine and L-arginine.

(19) DNase : Negative

(20) N-Acyl type of cell wall : Acetyl

(21) Main diamino acid of cell wall : Lysine

(22) Mol % of guanine (G) plus cytosine (C) of DNA : 71.2%

These bacteriological properties were compared with those of known microorganisms with reference to *Bergey's Manual of Systematic Bacteriology*, Vol. 2 (1984). As a result, it was revealed that the microorganism was identified as a novel one of the genus *Arthrobacter*. Based on the results, the present inventors named this microorganism "*Arthrobacter* sp. S34". The microorganisms was deposited and accepted on August 6, 1998, under the accession number of FERM BP-6450 in and by the Patent Microorganism Depository, National Institute of Bioscience and Human-Technology Agency of Industrial Science & Technology, Ministry of International Trade & industry, 1-3, Higashi, 1 chome, Tsukuba-shi, Ibaraki-ken 305-8566, Japan.

The homology of DNA between the identified microorganism and type-strains of the genus *Arthrobacter*, deposited in American Type Culture Collection (ATCC), an international depository of microorganism in USA, was examined in accordance with the DNA-DNA hybridization method in *Bergey's Manual of Systematic Bacteriology*, Vol.1 (1984). Twelve type-strains shown in Table 1 in the below were respectively cultured in a usual manner, and proliferated cells were collected from the resulting cultures. *Arthrobacter* sp. S34, FERM BP-6450, was cultured by the seed culture method in the later described Example 2-1, followed by collecting the proliferated cells. According to conventional method, DNAs were obtained from each type-strain of microorganisms, two micrograms aliquots of the DNAs were digested with a restriction enzyme, *Pst* I. The resulting digested mixtures were respectively spotted on "**Hybond-N+**", a nylon membrane commercialized by Amersham International, Arlington Heights, IL, USA, and in a usual manner, treated with alkali, neutralized, and dried to fix the DNAs on the nylon membrane. One microgram of the DNA obtained from *Arthrobacter* sp. S34, FERM BP-6450, was provided and digested with *Pst* I. Using [α-^{32}P] dCTP commercialized by Amersham International, Arlington Heights, IL, USA, and "**READY-TO-GO DNA-LABELLING KIT**", a DNA-labelling kit commercialized by Pharmacia LKB Biotechnology AB, Uppsala, SWeden, the digestant was labelled with an isotope to obtain a probe. The probe and the above DNA fixed on nylon film were hybridized for two hours

under shaking conditions at 65°C in "**RAPID HYBRIDIZATION BUFFER**", a buffer for hybridization commercialized by Amersham Corp., Div., Amersham International, Arlington Heights, IL, USA. The nylon film after hybridization was washed in a usual manner, dried and subjected to autoradiography in a usual manner. Signals of hybridization observed on radiography were analyzed on "**IMAGE MASTER**", an image analyzing system commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, followed by expressing numerically the intensity of the signals for hybridization. Based on the numerals, the relative intensities (%) of spots for the DNAs derived from the type-strains were calculated by regarding the signal intensity of a spot for the DNA from *Arthrobacter* sp. S34, FERM BP-6450, as 100 and used as an index for the DNA homology between the microorganism and the type-strains. The results are in Table 1.

Table 1

Strain of microorganism	Signal intensity of hybridization
Arthrobacter atrocyaneus, ATCC 13752	42.0
Arthrobacter aurescens, ATCC 13344	12.4
Arthrobacter citreus, ATCC 11624	36.2
Arthrobacter crystallpoietes, ATCC 15481	31.6
Arthrobacter globiformis, ATCC 8010	55.1
Arthrobacter nicotianae, ATCC 15236	18.8
Arthrobacter oxydans, ATCC 14358	28.3
Arthrobacter pascens, ATCC 13346	24.6
Arthrobacter protophormiae, ATCC 19271	29.3
Arthrobacter ramosus, ATCC 13727	98.6
Arthrobacter ureafaciens, ATCC 7562	42.3
Arthrobacter viscous, ATCC 19584	0.0
Arthrobacter sp. S34, FERM BP-6450	100

As shown in Table 1, the signal intensity of hybridization for the spot of DNA from *Arthrobacter ramosus* type strain, ATCC 13727, was as high as 98.6%. The data revealed that *Arthrobacter* sp. S34, FERM BP-6450, had the highest homology with *Arthrobacter ramosus* type-strain, ATCC 13727, among the 12 type strains used in this Example. The results in the above shows that *Arthrobacter* sp. S34, FERM BP-6450, is a novel microorganism nearly related to *Arthrobacter ramosus* type-strain, ATCC 13727.

Example 2

Non-reducing saccharide-forming enzyme

Experiment 2-1

Preparation of enzyme

A nutrient culture medium, consisting of 1.0 w/v % "**PINE-DEX #4**", a dextrin commercialized by Matsutani Chemical Ind., Tokyo, Japan, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % monosodium phosphate, 0.06 w/v % dipotassium hydrogen phosphate, 0.05 w/v % magnesium sulfate, and water, was prepared and adjusted to pH 7.0. About 100 ml aliquots of the medium were placed in 500-ml Erlenmeyer flasks which were then autoclaved at 120°C for 20 min and cooled, followed by an inoculation of a seed of *Arthrobacter* sp. S34, FERM BP-6450 and a culture at 37°C for 48 hours under stirring conditions of 260 rpm for obtaining a seed culture.

Except for containing 0.05 w/v % of "**KM-75**", a antifoamer commercialized by Shin-Etsu Chemical, Co., Ltd,

Tokyo, Japan, an about 20 ℓ of the same nutrient culture medium as used in the seed culture was placed in a 30-ℓ fermenter, sterilized, cooled to 37°C, and inoculated with one v/v % of the seed culture to the medium, followed by an incubation at 37°C and pHs of 5.5-7.5 for about 72 hours under aeration-agitation conditions.

A portion of the resultant culture was sampled, centrifuged to separate into cells and a culture supernatant. The cells were ultrasonically disrupted and centrifuged to collect supernatant for a cell extract. Assay for non-reducing saccharide-forming enzyme activity in each culture supernatant and cell extract revealed that the former showed a relatively-low enzyme activity and the latter exhibited an about 0.1 unit with respect to one milliliter of the culture.

Example 2-2

Purification of enzyme

An about 80 ℓ of a culture, obtained according to the method in Example 2-1, was centrifuged at 8,000 rpm for 30 min to obtain an about 800 g cells by wet weight. The wet cells were suspended in two liters of 10 M phosphate buffer (pH 7.0) and treated with "**MODEL UH-600**", an ultrasonic homogenizer commercialized by SMT Co., Tokyo, Japan. The resulting solution was centrifuged at 10,000 rpm for 30 min to yield an about 2 ℓ of a culture supernatant. To and in the culture supernatant was added and dissolved ammonium sulfate to give a saturation degree of 0.7, and the mixture was allowed to stand at 4°C for 24 hours and centrifuged at 10,000 rpm for 30 min to obtain a

precipitate. The precipitate thus obtained was dissolved in 10 mM phosphate buffer (pH 7.0) and dialyzed against a fresh preparation of the same buffer as above for 48 hours, followed by centrifuging the dialyzed inner solution at 10,000 rpm for 30 min to remove insoluble substances. An about one liter of the resulting solution was subjected to an ion-exchange column chromatography using a column packed with about 1.3 ℓ of "**SEPABEADS FP-DA13 GEL**", an anion exchanger commercialized by Mitsubishi Chemical Industries Ltd., Tokyo, Japan. The elution step was carried out using a linear gradient buffer of 10 mM phosphate buffer (pH 7.0) containing salt which increased from 0 M to 0.6 M. The eluate from the column was fractionated, and the fractions were respectively assayed for non-reducing saccharide-forming enzyme activity. As a result, the enzyme activity was remarkably found in fractions eluted with buffer having a salt concentration of about 0.2 M, followed by pooling the fractions.

Ammonium sulfate was added to the resulting solution to give a concentration of 1 M, and the mixture was allowed to stand at 4°C for 12 hours, centrifuged at 10,000 rpm for 30 min to collect a supernatant. The supernatant thus obtained was subjected to hydrophobic column chromatography using a column packed with "**BUTYL TOYOPEARL 650M GEL**", a hydrophobic gel commercialized by Tosoh Corporation, Tokyo, Japan. The gel volume used was about 300 ml and used after equilibrated with 10 mM phosphate buffer (pH 7.0) containing 1 M ammonium sulfate. The elution step was carried out using a linear gradient buffer

of 10 mM phosphate buffer (pH 7.0) containing ammonium sulfate which decreased from 1 M to 0 M during the feeding. The eluate from the column was fractionated, and the fractions were respectively assayed for non-reducing saccharide-forming enzyme activity. As a result, the enzyme activity was remarkably found in fractions eluted with buffer having a salt concentration of about 0.75 M, followed by pooling the fractions.

The resulting solution was dialyzed against 10 mM phosphate buffer (pH 7.0), and the resulting dialyzed inner solution was centrifuged at 10,000 rpm for 30 min to collect a supernatant, followed by subjecting the supernatant to ion-exchange column chromatography using a column packed with about 40 ml of "**DEAE TOYOPEARL 650S GEL** ", an anion exchanger commercialized by Tosoh Corporation, Tokyo, Japan. The elution step was carried out using a linear aqueous salt solution which increased from 0 M to 0.2 M during the feeding. The eluate from the column was fractionated, and the fractions were respectively assayed for non-reducing saccharide-forming enzyme activity. As a result, the enzyme activity was remarkably found in fractions eluted with buffer having a salt concentration of about 0.15 M, followed by pooling the fractions. The resulting solution was further subjected to gel filtration column chromatography using a column packed with about 380 ml of "**ULTROGEL® AcA44 GEL**", a gel for gel filtration column chromatography commercialized by Sepracor/IBF s.a. Villeneuve la Garenne, France, followed by collecting fractions with the desired enzyme activity. The level of the non-reducing

saccharide-forming enzyme activity, specific activity, and yields in the above purification steps are in Table 2.

Table 2

Purification step	Enzyme activity of non-reducing saccharide-forming enzyme	Specific activity (unit/mg protein)	Yield (%)
Cell extract	8,000	-	100
Dialyzed inner-solution after salting out with ammonium salt	7,500	0.2	94
Eluate from **SEPABEADS** column	5,200	0.7	65
Eluate from hydrophobic column	2,600	6.3	33
Eluate from **TOYO PEARL**	910	67.4	11
Eluate of gel filtration	59.0	168	0.7

The solution eluted and collected from the above gel filtration chromatography was in a usual manner subjected to electrophoresis using 7.5 w/v % polyacrylamide gel and resulted in a single protein band. The data shows that the eluate from gel filtration chromatography was a purified specimen of a non-reducing saccharide-forming enzyme purified up to an electrophoretically homogeneous form.

Example 2-3

Property of enzyme

Example 2-3(a)

Action

A 20% aqueous solution containing glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose or maltoheptaose as a substrate for enzyme was prepared, mixed with two units/g substrate, d.s.b., of a purified specimen of a non-reducing saccharide-forming enzyme obtained by the method in Example 2-2, and enzymatically reacted at 50°C and pH 6.0 for 48 hours. The reaction mixture was desalted and analyzed on high-performance liquid chromatography (abbreviated as "HPLC" hereinafter) using two columns of "MCI GEL CK04SS COLUMN", commercialized by Mitsubishi Chemical Industries Ltd., Tokyo, Japan, which were cascaded in series, followed by determining the saccharide composition of the reaction mixture. The conditions and apparatus used in HPLC were as follows: The column was kept at 85°C using "CO-8020", a column oven commercialized by Tosoh Corporation, Tokyo, Japan. Water as a moving phase was fed at a flow rate of 0.4 ml/min. The eluate was analyzed on "RI-8020", a differential refractometer

commercialized by Tosoh Corporation, Tokyo, Japan. The results were in Table 3.

Table 3

Substrate	Reaction product	Elution time (min)	Percentage (%)
Glucose	Glucose	57.2	100.0
Maltose	Maltose	50.8	100.0
Maltotriose	Glucosyltrehalose Maltotriose	43.2 46.2	36.2 63.8
Maltotetraose	Maltosyltrehalose Maltotetraose	38.9 42.3	87.2 12.8
Maltopentaose	Maltotriosyltrehalose Maltopentaose	35.4 38.4	93.0 7.0
Maltohexaose	Maltotetraosyltrehalose Maltohexaose	32.7 35.2	93.8 6.2
Maltoheptaose	Maltopentaosyltrehalose Maltoheptaose	30.2 32.4	94.2 5.8

As evident form the results in Table 3, each reaction product consisted essentially of the remaining substrate and a newly formed non-reducing saccharide of α-glucosyltrehalose, α-maltosyltrehalose, α-maltotriosyltrehalose, α-maltotetraosyltrehalose, or α-maltopentaosyltrehalose (in Table 3, it is expressed as glucosyltrehalose, maltosyltrehalose, maltotriosyltrehalose, maltotetraosyltrehalose, or maltopentaosyltrehalose). Substantially no other saccharide was detected in the reaction mixture. Regarding and evaluating the percentage of non-reducing saccharide in each reaction product as a production yield, it was revealed that the yield of α-glucosyltrehalose having a glucose polymerization degree of 3 was relatively low and the yield of those having a glucose polymerization degree of 4 or higher such as α-maltosyltrehalose, α-maltotriosyltrehalose, α-maltotetraosyltrehalose, and α-maltopentaosyltrehalose was as high as about 85% or higher. No formation of non-reducing saccharide from glucose and maltose was observed.

Example 2-3(b)

Molecular weight

A purified specimen of a non-reducing saccharide-forming enzyme, obtained by the method in Example 2-2, was subjected to SDS-PAGE using 10 w/v % polyacrylamide gel in a usual manner in parallel with molecular markers commercialized by Japan Bio-Rad Laboratories, Tokyo, Japan. Comparing with the positions of the molecular markers after electrophoresis, the non-reducing saccharide-forming enzyme exhibited a molecular weight of about 75,000±10,000 daltons.

Example 2-3(c)

Isoelectric point

A purified specimen of a non-reducing saccharide-forming enzyme, obtained by the method in Example 2-2, was isoelectrophoresed using a polyacrylamide gel containing 2 w/v % "**AMPHOLINE**", an ampholyte, commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden. After isoelectrophoresis, the measurement of the pH of gel revealed that the non-reducing saccharide-forming enzyme had an isoelectric point of about 4.5±0.5.

Example 2-3(d)

Optimum temperature and pH

Using a purified specimen of a non-reducing saccharide-forming enzyme, obtained by the method in Example 2-2, it was examined the influence of temperature and pH on the activity of the non-reducing saccharide-forming enzyme. When examining the influence of temperature, it was conducted similarly as in the assay for enzyme activity except for reacting the enzyme at different temperatures. In the examination of the influence of pH, it was conducted similarly as in the assay for enzyme activity except for reacting the enzyme at different pHs using appropriate 20 mM buffers. In each examination, a relative value (%) of a lowered level of reducing power of substrate in each reaction system was calculated into its corresponding relative enzyme activity (%). FIG. 1 shows a result of the influence of temperature, and FIG. 2 is of pH. The cross axles in FIGs. 1 and 2 show reaction temperatures and reaction pHs, respectively. As shown in FIG.

1, the optimum temperature of the enzyme was about 50°C when incubated at pH 6.0 for 60 min. Also as shown in FIG. 2, the optimum pH of the enzyme was a pH of about 6.0 when incubated at 50°C for 60 min.

Example 2-3(e)

Thermal and pH stabilities

Using a purified specimen of a non-reducing saccharide-forming enzyme, obtained by the method in Example 2-2, it was examined the thermal and pH stabilities of the enzyme. The thermal stability was examined by diluting the specimen with 20 mM phosphate buffer (pH 7.0), incubating the dilutions at prescribed temperatures for 60 min, cooling the incubated dilutions, and determining the remaining enzyme activity in the dilutions according to the method of the assay for the enzyme activity. The pH stability of the enzyme was examined by diluting the specimen with 50 mM buffers with appropriate different pHs, incubating the dilutions at 4°C for 24 hours, adjusting the dilutions to pH 6, and determining the remaining enzyme activity in the dilutions according to the method of the assay for the enzyme activity. The results of the thermal and pH stabilities of the enzyme are respectively shown in FIGs. 3 and 4. The cross axles in FIGs. 3 and 4 show incubation temperatures and pHs for the enzyme, respectively. As shown in FIG. 3, the enzyme was stable up to about 55°C and was stable at pHs in the range of about 5.0 to about 10.0 as shown in FIG. 4.

These results evidence that the non-reducing saccharide forming-enzyme, obtained by the method in Example 2-

2, is the present non-reducing saccharide-forming enzyme having an optimum temperature in a medium temperature range.

Example 2-4

Partial amino acid sequence

A portion of a purified specimen of a non-reducing saccharide-forming enzyme, obtained by the method in Example 2-2, was dialyzed against distilled water to obtain an about 80 μg of a sample by weight as a protein for analyzing the N-terminal amino acid sequence. Using "**PROTEIN SEQUENCER MODEL 473A**", a protein sequencer commercialized by Applied Biosystems, Inc., Foster City, USA, the N-terminal amino acid sequence was analyzed up to 20 amino acid residues from the N-terminus. The revealed N-terminal amino acid sequence was the partial amino acid sequence of SEQ ID NO:4. A portion of a purified specimen of a non-reducing saccharide-forming enzyme, obtained by the method in Example 2-2, was dialyzed against 10 mM Tris-HCl buffer (pH 9.0) and in a usual manner concentrated up to an about one mg/ml solution using "**ULTRACENT-30**", an ultrafiltration membrane commercialized by Tosoh Corporation, Tokyo, Japan. To 0.2 ml of the concentrate was added 10 μg "**TPCK-TRYPSIN**", a reagent trypsin commercialized by Wako Pure Chemical Industries, Ltd., Tokyo, Japan, allowed to react at 30°C for 22 hours to digest the enzyme to form peptides. The peptides were separated by subjecting the reaction mixture to reverse-phase HPLC using "**μ BONDASPHERE C18 COLUMN**" having a diameter of 3.9 mm and a length of 150 mm, a product of Waters Chromatography Div., MILLIPORE Corp., Milford, USA. The elution step was carried out at ambient temperature by feeding to the

column an aqueous solution containing 0.1 v/v % trifluoro acetate and acetonitrile increasing from 24 to 48 v/v % for 60 min during the feeding at a flow rate of 0.9 ml/min. The peptides eluted from the column were detected by monitoring the absorbance at a wavelength of 210 nm. Two peptides, which were well separated from others, i.e., "**S5**" eluted at a retention time of about two hours and "**S8**" eluted at a retention time of about 30 min were separated, respectively dried *in vacuo*, and dissolved in 50 v/v % aqueous acetonitrile solutions containing 50 µl of 0.1 v/v % trifluoro acetate. The peptide solutions were subjected to the protein sequencer to analyze up to 20 amino acid residues. From peptides "**S5**" and "**S8**" the amino acid sequences of SEQ ID NOs:5 and 6 were obtained.

Example 3

DNA encoding non-reducing saccharide-forming enzyme

Example 3-1

Construction and screening of gene library

Except for setting temperature and time for culture were respectively set to 27°C and 24 hours, *Arthrobacter* sp. S34, FERM BP-6450, was cultured similarly as in Example 2-1.

The culture was centrifuged to remove cells which were then suspended in an adequate amount of Tris-EDTA-salt bufferred saline (hereinafter designated as "**TES buffer**") (pH 8.0), admixed with lysozyme in an amount of 0.05 w/v % to the cell suspension by volume, followed by an incubation at 37°C for 30 min. The resultant mixture was freezed by standing at -80°C for one hour, and then admixed and sufficiently stirred with a mixture of TES buffer and phenol preheated to 60°C, cooled, and

centrifuged to collect the formed supernatant. To the supernatant was added cold ethanol was added, and then the formed sediment was collected, dissolved in an adequate amount of SSC buffer (pH 7.1), admixed with 7.5 μg ribonuclease and 125 μg protease, and incubated at 37°C for one hour. The resulting mixture was admixed and stirred with chloroform/isoamyl alcohol, and allowed to stand, followed by collecting the formed upper layer, adding cold ethanol to the layer, and collecting the formed sediment. The sediment was rinsed with a cold 70 v/v ethanol, dried *in vacuo* to obtain a DNA, followed by dissolving in SSC buffer (pH 7.1) to give a concentration of about one mg/ml, and freezing at -80°C.

Fifty microliters of the DNA was provided, admixed with an abut 50 units of *Kpn*I as an restriction enzyme, and incubated at 37°C for one hour to digest the DNA. Three micrograms of the digested DNA and 0.3 microgram of "**pBluescript II SK (+)**", a plasmid vector commercialized by Stratagene Cloning Systems, California, USA, was weighed, subjected to the action , were ligated using "**DNA LIGATION KIT**", commercialized by Takara Shuzo Co., Ltd., Tokyo, Japan, according to the protocol affixed to the kit. According to conventional competent cell method, 100 μl of "**Epicurian Coli XL1-Blue**", an Escherichia coli strain commercialized by Stratagene Cloning Systems, California, USA, was transformed with the ligated product. Thus a gene library was obtained.

The gene library thus obtained was inoculated to a agar nutrient plate medium (pH 7.0) containing 10 g/ℓ tryptone, 5 g/ℓ yeast extract, 5 g/ℓ sodium chloride, 75 mg ampicillin

sodium salt, and 50 mg/ℓ 5-bromo-4-chloro-indolyl-β-galactoside, and incubated at 37°C for 18 hours. About 5,000 white colonies formed on the medium were in a usual manner fixed on "**HYBOND-N+**", a nylon film commercialized Amersham Corp., Div. Amersham International, Arlington Heights, IL, USA. Based on 1-8 amino acid residues in the amino acid sequence of SEQ ID NO:5 revealed in Example 2-4, an oligonucleotide having the nucleotide sequence of SEQ ID NO:18 was chemically synthesized, and in a usual manner labelled with [γ-^{32}P] ATP and T4 polynucleotide kinase to obtain a probe. Using the probe, the colonies, which had been fixed on the nylon film and obtained previously, were screened by conventional colony hybridization method. The hybridization was carried out at 65°C for 16 hours in a solution for hybridization containing 6 x SSC, 5 x Denhalt solution, and 100 mg/ℓ of denatured salmon sperm DNA. The above nylon film after the hybridization was washed with 6 x SSC at 65°C for 30 min, and further washed with 2 x SSC containing 0.1 w/v % SDS at 65°C for two hours. The resulting nylon film was in a usual manner subjected to autoradiography, and then, based on the signals observed on the autoradiography, a colony which strongly hybridized with the probe was selected and named "GY1" as a transformant.

Example 3-2

Decoding of nucleotide sequence

According to conventional manner, the transformant GY1 was inoculated to L-broth (pH 7.0) containing 100 μg/ml ampicillin in a sodium form, and cultured at 37°C for 24 hours under shaking conditions. After completion of the culture, the

proliferated cells were collected from the culture by centrifugation and treated with conventional alkali-SDS method to extract a recombinant DNA. The recombinant DNA was named pGY1. Using the above probe, the recombinant DNA, pGY1, was analyzed on conventional Southern blot technique, and based on the analytical data a restriction map was constructed as shown in FIG. 5. As shown in FIG. 5, it was revealed that the recombinant DNA, pGY1, contained a nucleotide sequence consisting of bases of about 5,500 base-pairs (bp) from *Arthrobacter* sp. S34, FERM BP-6450, expressed with a bold line, and that the recombinant DNA contained a nucleotide sequence encoding the present non-reducing saccharide-forming enzyme, as indicated with a black arrow within the area of the bold line, in the area consisting bases of about 4,000 bp between two recognition sites by a restriction enzyme, *Eco*RI. Based on the result, the recombinant DNA, pGY1, was completely digested with *Eco*RI, and then a DNA fragment of about 4,000 bp was separated and purified using conventional agarose gel electrophoresis. The DNA fragment and "**pBluescript II SK (+)**", a plasmid vector commercialized by Stratagene Cloning Systems, California, USA, which had been previously digested with *Eco*RI, were ligated with conventional ligation method. With the ligated product, "**XL1-BLUE**", an *Escherichia coli* strain commercialized by Stratagene Cloning Systems, California, USA, was transformed to obtain a transformant. A recombinant DNA was extracted from the transformant in a usual manner, confirming in a usual manner that it contained the aforesaid DNA fragment consisting of about

bases of 4,000 bp, and named it "**pGY2**". The transformant introduced with "**pGY2**" was named "**GY2**".

The analysis of the nucleotide sequence of the recombinant DNA pGY2 on conventional dideoxy method revealed that it contained the nucleotide sequence of SEQ ID NO:19 consisting bases of 3252 bp derived from *Arthrobacter* sp. S34, FERM BP-6450. The nucleotide sequence encodes the amino acid sequence as shown in parallel in SEQ ID NO:19. Comparing the amino acid sequences of SEQ D NOs:4 to 6 as partial amino acid sequences of the present non-reducing saccharide-forming enzyme confirmed in Example 2-4, the amino acid sequences of SEQ ID NOs:4 to 6 were perfectly coincided with the amino acids 2-21, 619-638, and 98-117 in SEQ ID NO:19. These data indicate that the present non-reducing saccharide-forming enzyme obtained in Example 2 consists of the amino acids 2-757 of SEQ ID NO:19, or has the amino acid SEQUence of SEQ ID NO:1, and that the enzyme of *Arthrobacter* sp. S34, FERM BP-6450, is encoded by a nucleotide sequence of bases 746-3013 of SEQ ID NO:19, or encoded by the nucleotide sequence of SEQ ID NO:7. The structure of the recombinant DNA pGY2 is in FIG. 6.

The above-identified amino acid sequence of the present non-reducing saccharide-forming enzyme obtained by the method in Example 2, and amino acid sequences of known enzymes having a non-reducing saccharide-forming activity were compared using "**GENETYX-MAC, VER. 8**", a commercially available computer program commercialized by Software Development Co., Ltd., Tokyo, Japan, according to the method by Lipman, David J. in *Science*, Vol. 227, pp. 1,435-1,441 (1985) to calculate their homology

(%). The enzymes used as known enzymes were those from *Arthrobacter* sp. Q36 and *Rhizobium* sp. M-11 disclosed in Japanese Patent Kokai No. 322,883/95; *Sulfolobus acidocaldarius*, ATCC 33909, disclosed in Japanese Patent Kokai No. 84,586/96; and *Sulfolobus solfataricus* KM1 disclosed in Sai-Kohyo No. WO 95/34642. As disclosed in the above publications, the conventional enzymes have optimum temperatures other than a medium temperature range. The information of amino acid sequences of conventional enzymes is obtainable from the GeneBank, a DNA database produced by the National Institutes of Health (NIH), USA, under the accession numbers of D63343, D64128, D78001 and D83245. The obtained homologies are in Table 4.

Table 4

Origin of enzyme for amino acid sequence(*) comparison	Homology on amino acid sequence
Rhizobium sp. M-11 (D78001)	56.9%
Arthrobacter sp. Q36 (D63343)	56.6%
Sulfolobus solfataricus KM1 (D64128)	33.2%
Sulfolobus acidocaldarius, ATCC 33909 (D83245)	31.4%

* : Numerals in parentheses are access numbers to the GeneBank.

As shown in Table 4, the present non-reducing saccharide-forming enzyme in Example 2 showed a highest amino acid homology of 56.9% with the enzyme from *Rhizobium* sp. M-11 among conventional enzymes with optimum temperatures out of a

medium temperature range. The data indicates that the present non-reducing saccharide-forming enzyme generally comprises an amino acid sequence with a homology of at least 57% with the amino acid sequence of SEQ ID NO:1. The comparison result on amino acid sequence revealed that the enzyme in Example 2 and the above-identified four types of conventional enzymes have common amino acid sequences of SEQ ID NOs:2 and 3. The enzyme in Example 2 has partial amino acid sequences of SEQ ID NOs:2 and 3 as they correspond to amino acids 84-89 and 277-282 in SEQ ID NO:1. The four types of enzymes used as references have the above partial amino acid sequences which are positioned at their corresponding parts. Based on the fact that any of the present enzyme in Example 2 and the enzymes as references have a common activity of forming non-reducing saccharides having a trehalose structure as an end unit from reducing partial starch hydrolysates, it was indicated that the partial amino acid sequences of SEQ ID NOs:2 and 3 correlated to the expression of such an enzyme activity. These results show that the present non-reducing saccharide-forming enzyme can be characterized in that it comprises the amino acid sequences of SEQ ID NOs:2 and 3, and has an optimum temperature in a medium temperature range.

Example 3-3

Transformant introduced with DNA

Based on the 5'- and 3'-termini of the nucleotide sequence of SEQ ID NO:7, an oligonucleotide of the nucleotide sequences of SEQ ID NOs:20 and 21 were chemically synthesized in a usual manner. As sense- and anti-sense-primers, 85 ng of each of the oligonucleotide and 100 ng of the recombinant DNA

pGY2 in Example 3-2 as a template were mixed in a reaction tube, and the mixture was admixed with 1.25 units of "**PYROBEST**", a thermostable DNA polymerase specimen commercialized by Takara Shuzo Co., Ltd., Tokyo, Japan, together with 5 μl of a buffer affixed with the specimen and 4 μl of a dNTP mixture. The resulting mixture was brought up to a volume of 50 μl with sterilized distilled water to effect PCR. The temperature for PCR was controlled in such a manner that the mixture was treated with 25 cycles of successive incubations of 95°C for one minute, 98°C for 20 seconds, 70°C for 30 seconds, and 72°C for four minutes, and finally incubated at 72°C for 10 min. A DNA as a PCR product was collected in a usual manner to obtain an about 2,300 bp DNA. The DNA thus obtained was admixed with "**pKK223-3**", a plasmid vector commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, which had been previously cleaved with a restriction enzyme, *Eco*RI, and blunted by "**DNA BLUNTING KIT**" commercialized by Takara Shuzo Co., Ltd., Tokyo, Japan, and ligated by conventional ligation method. Thereafter, the ligated product was treated in a usual manner to obtain a recombinant DNA introduced with the above DNA consisting of bases of about 2,300 bp. Decoding of the recombinant DNA showed that it comprised a nucleotide sequence which two nucleotide sequences of 5'-ATG-3' and 5'-TGA-3' were respectively added to the 5'- and 3'-termini of the nucleotide sequence of SEQ ID NO:7. The DNA was named "**pGY3**". The structure of the recombinant DNA pGY3 was in FIG. 7.

The recombinant DNA pGY3 was in a usual manner introduced into an *Escherichia coli* LE 392 strain, ATCC 33572,

which had been competented in conventional manner, to obtain a transformant. Conventional alkali-SDS method was applied for the transformant to extract a DNA, and then the extracted DNA was confirmed to be pGY3 in a usual manner and named "**GY3**". Thus a transformant introduced with a DNA encoding the present non-reducing saccharide-forming enzyme.

Example 3-4

Transformant introduced with DNA

Based on a nucleotide sequence in the downstream of the 3'-terminus of a promotor in "**pKK223-3**", a plasmid vector commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, oligonucleotide having the nucleotide sequences of SEQ ID NOs:22 and 23 were synthesized in conventional manner, and phosphorylated their 5'-termini using T4 polynucleotide kinase. The phosphorylated oligonucleotide were annealed, ligated with "**pKK223-3**", a plasmid vector commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, which had been previously cleaved with restriction enzymes of *Eco*RI and *Pst*I, by conventional ligation method. According to conventional method, the ligated product was introduced into an *Escherichia coli* strain which was then cultured and treated with alkali-SDS method to extract a DNA. The DNA thus obtained had a similar structure to a plasmid vector "**pKK223-3**", and had recognition sites by restriction enzymes of *Eco*RI, *Xba*I, *Spe*I, and *Pst*I at the downstream of the promotor. The present inventors named the DNA a plasmid vector "**pKK4**".

Similarly as in Example 3-3, PCR was done except for using oligonucleotide with the nucleotide sequences of SEQ ID

NOs:24 and 25, which had been chemically synthesized based on the 5'- and 3'-terminal partial nucleotide sequences of SEQ ID NO:7. A DNA as a PCR product was collected in a usual manner to obtain an about 2,300 bp DNA. The DNA thus obtained was cleaved with restriction enzymes, *Xba*I and *Spe*I, and the above plasmid vector pKK4, which had been cleaved with *Xba*I and *Spe*I, were ligated by conventional ligation method. Thereafter, the ligated product was treated in a usual manner to obtain a recombinant DNA with the nucleotide sequence of SEQ ID NO:7. The recombinant DNA was named "**pKGY1**".

Using overlap extension method, which two steps PCR were applied for and reported by Horthon, Robert M. in *Methods in Enzymology*, Vol. 217, pp. 270-279 (1993), a nucleotide sequence in the upper part of the 5'-terminus of SEQ ID NO:7 in the above DNA pKGY1 was modified. PCR as a first step PCR-A was done similarly as in Example 3-3 except for using, as sense- and anti-sense-primers, oligonucleotide of the nucleotide sequences of SEQ ID NOs:26 and 27, which had been chemically synthesized based on the nucleotide sequence of plasmid vector pKK4; and as a template the above recombinant DNA pKGY1. In parallel, PCR as a first step PCR-B was done similarly as in Example 3-3 except for using, as sense- and anti-sense-primers, oligonucleotide of the nucleotide sequences of SEQ ID NOs:28 and 29, which had been respectively chemically synthesized in a usual manner based on the nucleotide sequence of SEQ ID NO:7; and as a template the above recombinant DNA pKGY1. A DNA as a product of the first step PCR-A was collected in a usual manner to obtain an about 390 bp DNA. A DNA as a product in the first

stp PCR-B was collected in conventional manner to obtain an about 930 bp DNA.

PCR, as a second step PCR-A, was done similarly as in Example 3-3 except for using as a template a DNA mixture, i.e., a product of the first PCR-A and the first step PCR-B; as a sense primer the oligonucleotide sequence of the nucleotide sequence of SEQ ID NO:26; and as an anti-sense primer the oligonucleotide of the nucleotide sequence of SEQ ID NO:30, which had been chemically synthesized in conventional manner based on the nucleotide sequence of SEQ ID NO:7. The DNA as a product in the PCR was collected in a usual manner to obtain an about 1,300 bp DNA.

The DNA as a product in the second PCR-A was cleaved with restriction enzymes of *Eco*RI and *Bsi*WI, and the formed DNA consisting of bases of about 650 bp was collected in a usual manner. An about 6,300 bp DNA, which was formed after cleavage of the above recombinant DNA pKGY1 with restriction enzymes of *Eco*RI and *Bsi*WI, was collected in conventional manner. These DNAs were ligated in a usual manner, and the ligated product was treated in conventional manner to obtain a recombinant DNA comprising an about 650 bp DNA derived from the second step PCR-A. Decoding of the DNA by conventional dideoxy method revealed that the obtained recombinant DNA comprised a nucleotide sequence which the nucleotide sequence of SEQ ID NO:8, a nucleotide sequence represented by 5'-ATG-3', and a nucleotide sequence represented by 5'-TGA-3' were cascaded in the order as indicated above from the 5'-terminus to the 3'-terminus. The recombinant DNA thus obtained was named "**pGY4**". The structure

of pGY4 is substantially the same as the recombinant DNA pGY3 except for that pGY4 comprises the nucleotide sequence of SEQ ID NO:8.

The recombinant DNA pGY4 was introduced in conventional manner with "**BMH71-18mutS**", an *Escherichia coli* competent cell commercialized by Takara Shuzo Co., Ltd., Tokyo, Japan to obtain a transformant. The transformant was treated with alkali-SDS method to extract a DNA which was then identified with pGY4 in conventional manner. Thus a transformant introduced with a DNA encoding the present non-reducing saccharide-forming enzyme.

Example 4

Preparation of non-reducing non-reducing saccharide-forming enzyme

Example 4-1

Preparation of enzyme using microorganism of the genus *Arthrobacter*

In accordance with the method in Example 2-1, *Arthrobacter* sp. S34, FERM BP-6450, was cultured by a fermenter for about 72 hours. After the cultivation, the resulting culture was concentrated with an SF-membrane to yield an about eight liters of a cell suspension. The cell suspension was treated with "**MINI-LABO**", a supper high-pressure cell disrupter commercialized by Dainippon Pharmaceutical Co., Ltd., Tokyo, to disrupt the cells. The resulting solution was centrifuged to obtain an about 8.5 ℓ of a supernatant. When measured for non-reducing saccharide-forming activity in the supernatant, it showed an about 0.1 unit of the enzyme activity with respect to

one milliliter of the culture. Ammonium sulfate was added to the supernatant to brought up to a saturation degree of about 0.7 to salt out, and the sediment was collected by centrifugation, dissolved in 10 mM phosphate buffer (pH 7.0), and dialyzed against a fresh preparation of the same buffer. Except for using an about 2 ℓ of an ion-exchange resin, the resulting dialyzed inner solution was fed to ion-exchange column chromatography using "**SEPABEADS FP-DA13 GEL**", an anion exchanger commercialized by Mitsubishi Chemical Industries Ltd., Tokyo, Japan, as described in Example 2-2, to collect fractions with non-reducing saccharide-forming enzyme. The fractions were pooled, dialyzed against a fresh preparation of the same buffer but containing 1 M ammonium sulfate, and the resulting dialyzed inner solution was centrifuged to collect the formed supernatant. Except for using an about 300 ml gel, the supernatant was fed to hydrophobic column chromatography in accordance with the method described in Example 2-2 to collect fractions with non-reducing saccharide-forming enzyme. Then it was confirmed that the obtained enzyme had an optimum temperature over 40°C but below 60°C, i.e., a temperature in a medium temperature range, and an acid pH range of less than 7.

Thus an about 2,600 units of the present non-reducing saccharide-forming enzyme was obtained.

Example 4-2

Preparation of enzyme using transformant

One hundred ml of an aqueous solution containing 16 g/ℓ polypeptone, 10 g/ℓ yeast extract, and 5 g/ℓ sodium chloride was placed in a 500-ml Erlenmeyer flask, autoclaved at 121°C for

15 min, cooled, adjusted aseptically to pH 7.0, and admixed aseptically with 10 mg of ampicillin in a sodium salt to obtain a liquid nutrient medium. The nutrient medium was inoculated with the transformant GY2 in Example 3-2, and incubated at 37°C for about 20 hours under aeration-agitation conditions to obtain a seed culture. Seven liters of a medium having the same composition as used in the seed culture was prepared as in the case of the seed culture and placed in a 10-ℓ fermenter, and inoculated with 70 ml of the seed culture, followed by the incubation for about 20 hours under aeration-agitation conditions. From the resultant culture cells were collected by centrifugation in a usual manner. The collected cells were suspended in phosphate buffer (pH 7.0), disrupted by the treatment of ultrasonication, and centrifuged to remove insoluble substances, followed by collecting a supernatant to obtain a cell extract. The extract was dialyzed against 10 mM phosphate buffer (pH 7.0). The resulting dialyzed inner solution was collected and confirmed that it exhibited a non-reducing saccharide-forming enzyme activity, had an optimum temperature in a medium temperature range, i.e., a temperature of over 40°C but below 60°C, and had an optimum pH in an acid pH range, i.e., a pH of less than 7.

Thus the present non-reducing saccharide-forming enzyme was obtained. In the culture of this example, an about 0.2 unit/ml culture of the enzyme was produced.

As a control, "**XL1-BLUE**", an *Escherichia coli* strain commercialized by Stratagene Cloning Systems, California, USA, was cultured under the same conditions as above in a nutrient

culture medium of the same composition as used in the above except that it contained no ampicillin. Similarly as above, a cell extract was obtained and dialyzed. No activity of non-reducing saccharide-forming enzyme was detected in the resulting dialyzed inner solution, meaning that the transformant GY2 is useful in producing the present non-reducing saccharide-forming enzyme.

Example 4-3

Preparation of enzyme using transformant

The transformant GY3 in Example 3-3 was cultured similarly as in Example 4-2 except for using a liquid nutrient culture medium consisting of one w/v % maltose, three w/v polypeptone, one w/v % "**MEAST PIG**", a product of Asahi Breweries, Ltd., Tokyo, Japan, 0.1 w/v % dipotassium hydrogen phosphate, 100 μg/ml ampicillin, and water. The resultant culture was treated with ultrasonication to disrupt cells, and the resulting mixture was centrifuged to remove insoluble substances. When assayed for non-reducing saccharide-forming enzyme activity in the resulting supernatant, the culture contained about 15 units/ml culture of the enzyme. In accordance with the method in Example 2-2, the enzyme in the supernatant was purified, confirming that the resulting purified specimen exhibited a non-reducing saccharide-forming enzyme activity, had an optimum temperature in a medium temperature range, i.e., a temperature of over 40°C but below 60°C, and had an optimum pH in an acid pH range, i.e., a pH of less than 7. Thus the present non-reducing saccharide-forming enzyme was obtained.

Example 4-4

Preparation of enzyme using transformant

The transformant GY4 in Example 3-4 was cultured similarly as in Example 4-2 except for using a liquid nutrient culture medium consisting of two w/v % maltose, four w/v % peptone, one w/v % yeast extract, 0.1 w/v % sodium dihydrogen phosphate, 200 μg/ml ampicillin, and water. The resultant culture was treated with ultrasonication to disrupt cells, and the resulting mixture was centrifuged to remove insoluble substances. When assayed for non-reducing saccharide-forming enzyme activity in the resulting supernatant, the culture contained about 60 units/ml culture of the enzyme. In accordance with the method in Example 2-2, the enzyme in the supernatant was purified, confirming that the resulting purified specimen exhibited a non-reducing saccharide-forming enzyme activity, had an optimum temperature in a medium temperature range, i.e., a temperature of over 40°C but below 60°C, and had an optimum pH in an acid pH range, i.e., a pH of less than 7. Thus the present non-reducing saccharide-forming enzyme was obtained.

Example 5

Trehalose-releasing enzyme

Example 5-1

Production of enzyme

According to the method in EXample 2-1, *Arthrobacter* sp. S34, FERM BP-6450, was cultured by a fermenter. Then, in accordance with the method in Example 2-2, the resulting culture was sampled, followed by separating the sample into cells and

a supernatant. From the cells a cell extract was obtained. When assayed for trehalose-releasing activity of the supernatant and the cell extract, the former scarcely exhibited the enzyme activity, while the latter exhibited an about 0.3 uni/ml culture of the enzyme.

Example 5-2

Preparation of enzyme

An about 80 ℓ of a culture, prepared according to the method in Example 2-1, was centrifuged at 8,000 rpm for 30 min to obtain an about 800 g cells by wet weight. Two ℓ of the wet cells was suspended in 10 mM phosphate buffer (pH 7.0) and treated with "**MODEL UH-600**", an ultrasonic homogenizer commercialized by MST Co., Tokyo, Japan. The resulting suspension was centrifuged at 10,000 rpm for 30 min, followed a collection of an about two liters of a supernatant. The supernatant was admixed with ammonium sulfate to bring to a saturation degree of 0.7, allowed to stand at 4°C for 24 hours, and centrifuged at 10,000 rpm for 30 min to obtain a precipitate salted out with ammonium sulfate. The precipitate was dissolved in 10 mM phosphate buffer (pH 7.0), dialyzed against a fresh preparation of the same buffer for 48 hours, and centrifuged at 10,000 rpm for 30 min to remove insoluble substances. An about one liter of the resulting dialyzed inner solution was fed to ion-exchange column chromatography using an about 1.3 ℓ of "**SEPABEADS FP-DA13 GEL**", an anion exchanger commercialized by Mitsubishi Chemical Industries Ltd., Tokyo, Japan. The elution step was carried out using a linear 10 mM phosphate buffer (pH 7.0) containing salt decreasing from 0 M to 0.6 M during the

feeding. The eluate from the column was fractionated, and the fractions each were assayed for trehalose-releasing enzyme activity. As a result, the enzyme activity was remarkably found in fractions eluted with buffer having a salt concentration of about 0.2 M, followed by pooling the fractions.

Ammonium sulfate was added to the pooled solution to bring to a concentration of 1 M, and the mixture was allowed to stand at 4°C for 12 hours, centrifuged at 10,000 rpm for 30 min to collect a supernatant. The supernatant was subjected to hydrophobic column chromatography using a column packed with "**BUTYL TOYOPEARL 650M GEL**", a hydrophobic gel commercialized by Tosoh Corporation, Tokyo, Japan. Prior to use, the gel volume was set to about 300 ml and equilibrated with 10 mM phosphate buffer (pH 7.0) containing 1 M ammonium sulfate. The elution step was carried out using a linear gradient aqueous solution of ammonium decreasing from 1 M to 0 M during the feeding. The eluate from the column was fractionated, and the fractions were respectively assayed for trehalose-releasing enzyme activity. As a result, the enzyme activity was remarkably found in fractions eluted with buffer having an ammonium concentration of about 0.5 M, followed by pooling the fractions.

The fractions were pooled, dialyzed against 10 mM phosphate buffer (pH 7.0), and the dialyzed inner solution was centrifuged at 10,000 rpm for 30 min. Then the resulting supernatant was collected and subjected to "**DEAE-TOYOPEARL 650S GEL**", an anion exchanger commercialized by Tosoh Corporation, Tokyo, Japan. The elution step was carried out using a linear gradient aqueous solution of salt increasing from 0 M to 0.2 M

during the feeding. The eluate from the column was fractionated, and the fractions were respectively assayed for trehalose-releasing enzyme activity. As a result, the enzyme activity was remarkably found in fractions eluted with buffer having an ammonium concentration of about 0.15 M, followed by pooling the fractions. The pooled solution was subjected to gel filtration chromatography using about 380 ml of "**ULTROGEL® AcA44 RESIN**", a gel for gel filtration column chromatography commercialized by Sepracor/IBF s.a. Villeneuve la Garenne, France, followed collecting fractions with a remarkable activity of the enzyme. The content, specific activity, and yield of the enzyme in each step are in Table 5.

Table 5

Step	Activity of Trehalose-releasing enzyme (unit)	Specific activity (mg/ protein)	Yield (%)
Cell extract	24,000	-	100
Dialyzed inner solution after salting out with ammonium sulfate	22,500	0.6	94
Eluate from **SEPABEADS** column	15,600	2.0	65
Eluate from hydrophobic column	6,400	25.3	27
Eluate from **TOYOPEARL** column	4,000	131	17
Eluate after gel filtration	246	713	1.0

When electrophoresed in 7.5 w/v % polyacrylamide gel in conventional manner, the solution eluted and collected from the above gel filtration chromatography gave a single protein band. The data indicates that the eluate from gel filtration chromatography obtained in the above was a purified trehalose-releasing enzyme purified up to an electrophoretically homogeneous level.

Example 5-3

Property of enzyme

Example 5-3(a)

Action

Any one of saccharides consisting of α-glucosyltrehalose, α-maltosyltrehalose, α-maltotriosyltrehalose, α-maltotetraosyltrehalose, and α-maltopentaosyltrehalose as non-reducing saccharides having a trehalose structure obtained by the method in the later described Example 8-3; and maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose as reducing saccharides was dissolved in water into a 2 w/v % solution as an aqueous substrate solution for substrate. Each aqueous substrate solution was admixed with two units/g substrate, d.s.b., of a purified specimen of trehalose-releasing enzyme obtained by the method in Example 5-2, and enzymatically reacted at 50°C and pH 6.0 for 48 hours. In accordance with the method in Example 2-3(a), the reaction product was analyzed on HPLC after desalting to calculate the saccharide composition of the reaction products each. The results are in Table 6. In Table 6, α-glucosyltrehalose, α-maltosyltrehalose, α-maltotriosyltrehalose, α-maltotetraosyltrehalose, and α-

maltopentaosyltrehalose were respectively expressed as glucosyltrehalose, maltosyltrehalose, maltotriosyltrehalose, maltotetraosyltrehalose, and maltopentaosyltrehalose.

Table 6

Substrate	Reaction product	Elution time (min)	Composition (%)
Glucosyltrehalose	Trehalose	48.5	16.8
	Glucose	57.2	8.2
	Glucosyltrehalose	43.3	75.0
Maltosyltrehalose	Trehalose	48.5	44.1
	Maltose	50.8	44.4
	Maltosyltrehalose	38.9	11.5
Maltotriosyltrehalose	Trehalose	48.5	40.5
	Maltotriose	46.2	59.0
	Maltotriosyltrehalose	35.4	0.5
Maltotetraosyltrehalose	Trehalose	48.5	35.0
	Maltotetraose	42.1	64.2
	Maltotetraosyltrehalose	32.7	0.3
Maltopentaosyltrehalose	Trehalose	48.5	29.5
	Maltopentaose	38.2	70.2
	Maltopentaosyltrehalose	30.2	0.3
Maltotriose	Maltotriose	46.2	100.0
Maltotetraose	Maltotetraose	42.1	100.0
Maltopentaose	Maltopentaose	38.2	100.0
Maltohexaose	Maltohexaose	35.2	100.0
Maltoheptaose	Maltoheptaose	32.6	100.0

As evident from the results in Table 6, the trehalose-releasing enzyme, obtained by the method in Example 5-2, specifically hydrolyzed non-reducing a saccharide, which has a trehalose structure as an end unit and a glucose polymerization degree of at least three, at a site between a part of the trehalose structure and a part of the resting to form trehalose and a reducing saccharide having a glucose polymerization degree of one or more. While the enzyme did not act on maltooligosaccharides such as maltotriose and lower saccharides.

Example 5-3(b)

Molecular weight

A purified specimen of a trehalose-releasing enzyme, obtained by the method in Example 5-2, was subjected along with molecular markers commercialized by Japan Bio-Rad Laboratories, Tokyo, Japan, to conventional SDS-PAGE using 10 w/v % polyacrylamide gel. After electrophoresis, the position of the specimen electrophoresed on the gel was compared with those of the markers, revealing that the specimen had a molecular weight of about 62,000±5,000 daltons.

Example 5-3(c)

Isoelectric point

A purified specimen of a trehalose-releasing enzyme, obtained by the method in Example 5-2, was in a usual manner subjected to isoelectrophoresis using a polyacrylamide gel containing 2 w/v % "**AMPHOLINE**", an ampholyte commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden. Measurement of pH of the gel after electrophoresis, it had an isoelectric point of about 4.7±0.5.

Example 5-3(d)

Optimum temperature and pH

A purified specimen of a trehalose-releasing enzyme, obtained by the method in Example 5-2, was examined on the influence of the temperature and pH on the enzyme activity. The influence of temperature was examined according to the assay for enzyme activity except for reacting the enzyme at different temperatures. The influence of pH was examined according to the assay for enzyme activity except for reacting the enzyme at different pHs using appropriate 20 mM buffers. In each procedure, relative values (%) of the increased level of reducing power found in each system were calculated and regarded as relative enzyme activity (%). The results of the influence of temperature and pH are respectively in FIGs. 8 and 9. The cross axles in FIGs. 8 and 9 show reaction temperatures and pHs for the enzyme, respectively. As shown in FIG. 8, the optimum temperature of the enzyme was about 50 to about 55°C when incubated at pH 6.0 for 30 min, while the optimum pH of the enzyme was a pH of about 6.0 when incubated at 50°C for 30 min.

Example 5-3(e)

Stability on temperature and pH

A purified specimen of a trehalose-releasing enzyme, obtained by the method in Example 5-2, was examined on the stability of temperature and pH. The stability of temperature was examined by diluting the specimen with 20 mM phosphate buffer (pH 7.0), incubating the dilutions at different temperatures for 60 min, cooling the resulting dilutions, and assaying the enzyme activity remained in the dilutions. The pH

stability was studied by diluting the specimen with 50 mM buffers (pH 7.0) with different pHs, incubating the dilutions at 4°C for 24 hours, adjusted to pH 6, and assaying the enzyme activity remained in the dilutions. The results of the stability of temperature and pH are respectively in FIGs. 10 and 11. The cross axles in FIGs. 10 and 11 show temperatures and pHs at which the enzyme was kept, respectively. As shown in FIG. 10, the enzyme was stable up to about 50°C, while the enzyme was stable at pHs in the range of about 4.5 to about 10.0.

The results described hereinbefore indicate that the trehalose-releasing enzyme, obtained by the method in Example 5-2, is the present enzyme which has an optimum temperature in a medium temperature range.

Example 5-4

Partial amino acid sequence

A portion of a purified specimen of a trehalose-releasing enzyme, obtained by the method in Example 5-2, was dialyzed against distilled water and prepared into a sample containing about 80 ng protein for the N-terminal amino acid analysis. Using "**PROTEIN SEQUENCER MODEL 473A**", a protein sequencer commercialized by Applied Biosystems, Inc., Foster City, USA, the N-terminal amino acid sequence was analyzed up to 20 amino acid residues from the N-terminus. The revealed N-terminal amino acid sequence was the partial amino acid sequence of SEQ ID NO:14.

A portion of a purified specimen of a trehalose-releasing enzyme, obtained by the method in Example 5-2, was

dialyzed against 10 mM Tris-HCl buffer (pH 9.0) and in a usual manner concentrated to give a concentration of about one milligram per milliliter using "**ULTRACENT-30**", an ultrafiltration membrane commercialized by Tosoh Corporation, Tokyo, Japan. To 0.2 ml of the concentrate was added 10 µg of a lysyl endopeptidase reagent commercialized by Wako Pure Chemical Industries, Ltd., Tokyo, Japan, and the mixture was incubated at 30°C for 22 hours to digest the enzyme and to form peptides. The reaction mixture was subjected to reverse-phase HPLC using a column of "**NOVA-PAK C18 COLUMN**", 4.5 mm in diameter and 150 mm in length, commercialized by Waters Chromatography Div., Millipore Corp., Milford, MA, USA, to separate the peptides under ambient temperature. The elution step was carried out using a linear gradient of a 0.1 v/v % aqueous trifluoroacetic acid solution containing acetonitrile increasing from 24 v/v % to 48 v/v % for 60 min during the feeding at a flow rate of 0.9 ml/min. Peptides eluate from the column was monitored by measuring at a wavelength of 210 nm. Two peptides, named "**RT18**" with a retention time of about 18 min and "**RT33**" with a retention time of about 33 min and well separated from others, were collected, dried *in vacuo*, and dissolved respectively in a 50 v/v % aqueous acetonitrile solution containing 200 µl of 0.1 v/v % trifluoroacetic acid. The peptide solutions were subjected to a protein sequencer to analyze up to 20 amino acid residues from the N-terminus of each peptide. The amino acid sequences of SEQ ID NOs:15 and 16 from the peptides RT18 and RT33, respectively.

Example 6

DNA encoding trehalose-releasing enzyme

Example 6-1

Construction and screening of gene library

According to Example 3-1, a gene library of *Arthrobacter* sp. S34, FERM BP-6450 was constructed, and then subjected to screening by applying colony hybridization method under the conditions as used in Example 3-1 except for using as a probe an oligonucleotide, having a nucleotide sequence encoding the present trehalose-releasing enzyme, prepared by the following procedures; The probe was in a usual manner prepared by labelling with an isotope of [γ-^{32}P] ATP and T4 polynucleotide kinase the oligonucleotide having the nucleotide sequence of SEQ ID NO:31, which had been chemically synthesized based on an amino acid sequence consisting of amino acids 12-20 of SEQ ID NO:15 revealed in Example 5-4. A transformant which strongly hybridized with the prove was selected.

According to the method in Example 3-2, a recombinant DNA was extracted from the transformant and analyzed on conventional Southern blot technique using the above prove. A restriction map made based on the analytical data was coincided with that of the recombinant DNA pGY1 obtained in Examples 3-1 and 3-2. As shown in FIG. 5, it was revealed that the present recombinant DNA in this example contained a nucleotide sequence, which encoded the present trehalose-releasing enzyme as indicated with an oblique arrow, within a region consisting of bases of about 2,200 bp positioned between recognition sites by restriction enzymes, *Pst*I and *Kpn*I. Using the recombinant DNA pGY1, it was proceeded the decoding of the nucleotide sequence

of DNA encoding the present trehalose-releasing enzyme.

Example 6-2

Decoding of nucleotide sequence

The recombinant DNA pGY1, obtained by the method in Example 3-2, was in conventional manner completely digested with a restriction enzyme, *Pst*I. The DNA fragment of about 3,300 bp formed in the resulting mixture was removed on conventional agarose electrophoresis, and the formed DNA fragment of about 5,200 bp was collected. The DNA fragment was in a usual manner subjected to ligation reaction, and the ligated product was used to transform "**XL1-BLUE**", an *Escherichia coli* strain commercialized by Stratagene Cloning Systems, California, USA. From the resultant transformant, a recombinant DNA was extracted by conventional method. The recombinant DNA was confirmed to have a region consisting of bases of about 2,200 bp comprising a nucleotide sequence encoding the present trehalose-releasing enzyme, and named "**pGZ2**". A transformant intr254oduce with pGZ2 was named a recombinant DNA pGZ2.

Analysis of Conventional dideoxy method for the nucleotide sequence of the recombinant DNA pGZ2 revealed that it contained a nucleotide sequence consisting of 2,218 bp bases as shown in SEQ ID NO:32 derived from *Arthrobacter* sp. S34, FERM BP-6450. The nucleotide sequence could encode the amino acid sequence in SEQ ID NO:32. The amino acid sequence was compared with those of SEQ ID NOs:14 to 16 as partial amino acid sequences of the present trehalose-releasing enzyme confirmed in Example 5-4. As a result, the amino acid sequences of SEQ ID NOs:14, 15 and 16 were respectively coincided with amino

acids 1-20, 298-317, and 31-50 of the amino acid sequence in SEQ ID NO:32. The data indicates that the trehalose-releasing enzyme in Example 5 comprises the amino acid sequence in SEQ ID NO:32 or the one of SEQ ID NO:9, and that the enzyme from *Arthrobacter* sp. S34, FERM BP-6450, is encoded by bases 477-2,201 in SEQ ID NO:32 or the nucleotide sequence of SEQ ID NO:17. FIG. 12 shows the structure of the aforesaid recombinant DNA pGZ2.

The above amino acid sequence of the present trehalose-releasing enzyme, obtained by the method in Example 5, and other conventional ones of enzymes having an activity of trehalose-releasing enzyme were compared with each other in accordance with the method in Example 3-2 to determine their homology (%). As conventional enzymes, those derived from *Arthrobacter* sp. Q36 disclosed in Japanese Patent Kokai No. 298,880/95; *Rhizobium* sp. M-11, disclosed in Japanese Patent Kokai No. 298,880/95; *Sulfolobus acidocaldarius*, ATCC 33909; and Sulfolobus solfataricus KM1 disclosed in Sai-Kohyo No. WO95/34642. All of these enzymes have optimum temperatures out of a medium temperature range. The amino acid sequences of these enzymes are available from the GenBank, a DNA database produced by the National Institutes of Health (NIH), USA, under the accession numbers of D63343, D64130, D78001, and D83245. The information of their homology are in Table 7.

Table 7

Origin of enzyme for amino acid sequence(*) comparison	Homology on amino acid sequence
Arthrobacter sp. Q36 (D63343)	59.9%
Rhizobium sp. M-11 (D78001)	59.1%
Sulfolobus solfataricus KM1 (D64130)	37.7%
Sulfolobus acidocaldarius, ATCC 33909 (D83245)	36.0%

* : Numerals in parentheses are access numbers to the GeneBank.

As shown in Table 7, the present trehalose-releasing enzyme in Example 5 showed a highest amino acid homology of 59.9% with the enzyme from *Arthrobacter* sp. Q36 among conventional enzymes with optimum temperatures out of a medium temperature range. The data indicates that the present trehalose-releasing enzyme generally comprises an amino acid sequence with a homology of at least 60% with the amino acid sequence of SEQ ID NO:9. The comparison result on amino acid sequence revealed that the enzyme in Example 5 and the above-identified four types of conventional enzymes have common amino acid sequences of SEQ ID NOs:10 and 13. The enzyme in Example 5 has partial amino acid sequences of SEQ ID NOs:10 to 13 as found in amino acids 148-153, 185-190, 248-254 and 285-291 in SEQ ID NO:9. The four types of enzymes used as references have the above partial amino acid sequences which are positioned at their corresponding parts. Based on the fact that any of the present enzyme in Example 5 and the enzymes as references have commonly an activity of specifically hydrolysing a non-reducing

saccharide, which has a trehalose structure as an end unit and a glucose polymerization degree of at least three, at a site between a part of the trehalose structure and a part of the resting, it was indicated that the partial amino acid sequences of SEQ ID NOs:10 to 13 correlated to the expression of such an enzyme activity. These results show that the present trehalose-releasing enzyme can be characterized in that it comprises the amino acid sequences of SEQ ID NOs:10 to 13 and has an optimum temperature in a medium temperature range.

Example 6-3

Transformant introduced with DNA

Based on the 5'- and 3'-terminal nucleotide sequences of SEQ ID NO:17, oligonucleotides of the bases of SEQ ID NOs:33 and 34 were chemically synthesized in a usual manner. As sense- and anti-sense-primers, 85 ng of each of the oligonucleotides and 100 ng of the recombinant DNA pGZ2 in Example 6-2 as a template were mixed in a reaction tube while adding another reagents in accordance with Example 3-3. The temperature for PCR was controlled in such a manner that the mixture was treated with 25 cycles of successive incubations of 95°C for one minute, 98°C for 20 seconds, 70°C for 30 seconds, and 72°C for four minutes, and finally incubated at 72°C for 10 min. A DNA as a PCR product was collected in a usual manner to obtain an about 1,700 bp DNA. The DNA thus obtained was admixed with "**pKK233-3**", a plasmid vector commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, which had been previously cleaved with a restriction enzyme, *Eco*RI, and blunted by "**DNA BLUNTING KIT**" commercialized by Takara Shuzo Co., Ltd., Tokyo,

Japan, and ligated by conventional ligation method. Thereafter, the ligated product was treated in a usual manner to obtain a recombinant DNA introduced with the above DNA consisting of bases of about 1,700 bp. Decoding of the recombinant DNA by conventional dideoxy method showed that it comprised a nucleotide sequence which a nucleotide sequence of 5'-TGA-3' was added to 3'-terminus of the nucleotide sequence of SEQ ID NO:17. The DNA was named "**pGZ3**". The structure of the recombinant DNA pGZ3 was in FIG.13.

The recombinant pGZ3 was in a usual manner introduced into an *Escherichia coli* LE 392 strain, ATCC 33572, which had been competented in conventional manner, to obtain a transformant. Conventional alkali-SDS method was applied for the transformant to extract a DNA and named "**GZ3**" by identifying transformant as pGZ3. Thus a transformant, introduced with the present trehalose-releasing enzyme, was obtained.

Example 6-4

Transformant introduced with DNA

PCR was done similarly as in Example 6-3 except for using, as sense- and anti-sense-primers, oligonucleotide having nucleotide sequences of SEQ ID NOs:35 and 36, respectively, which had been chemically synthesized based on the 5'- and 3'-terminal nucleotide sequences of SEQ ID NO:17. A DNA as a PCR product was collected in a usual manner to obtain an about 1,700 bp DNA. The DNA thus obtained was cleaved with restriction enzymes, *Xba*I and *Spe*I, and "**pKK4**", a plasmid vector obtained by the method in Example 3-4, which had been previously cleaved with restriction enzyme, *Xba*I and *Spe*I, were ligated in a usual

manner. Thereafter, the ligated product was treated in a usual manner to obtain a recombinant DNA comprising the nucleotide sequence of SEQ ID NO:17. The recombinant DNA thus obtained was named "**pKGZ1**".

A nucleotide sequence in the upper part of the 5'-terminus of SEQ ID NO:17 contained in the recombinant DNA pKGZ1 was modified similarly as in Example 3-4; PCR as a first PCR-C was carried out similarly as in Example 3-3 except for using the above recombinant DNA pKGZ1 as a template and oligonucleotides of SEQ ID NOs:26 and 37, as sense- and anti-sense-primers, which had been chemically synthesized in a usual manner based on the nucleotide sequence of the plasmid vector pKK4. In parallel, PCR as a first PCR-D was carried out similarly as in Example 3-3 except for using the above recombinant DNA pKGZ1 as a template and oligonucleotides of SEQ ID NOs:38 and 39, as sense- and anti-sense-primers, which had been chemically synthesized in a usual manner based on the nucleotide sequences of SEQ ID NOs:38 and 39. A DNA as a PCR-C product was collected in a usual manner to obtain an about 390 bp DNA, while another DNA as a PCR-D product was collected similarly as above to obtain an about 590 bp DNA.

PCR as a second PCR-B was carried out similarly as in Example 3-3 except for using the DNA mixture obtained as products in the first PCR-C and first PCR-D, an oligonucleotide of SEQ ID NO:26 used in the first PCR-C as a sense primer, and an oligonucleotide of SEQ ID NO:39 used in the first PCR-D as an anti-sense primer. A DNA as a PCR product was collected in a usual manner to obtain an about 950 bp DNA.

The DNA as a second PCR-B product was cleaved with a restriction enzyme, *Eco*RI, and the formed about 270 bp DNA was collected in conventional manner. The recombinant DNA pKGZ1 was cleaved with a restriction enzyme, *Eco*RI, and the formed about 5,100 bp DNA was collected similarly as above. These DNAs were ligated as usual and treated in a usual manner to obtain a recombinant DNA comprising about 270 bp DNA from the second PCR-B product. Decoding of the recombinant DNA by conventional dideoxy method revealed that it contained the nucleotide sequence of SEQ ID NO:8, one of SEQ ID NO:17, and one represented by 5'-TGA-3' in the order as indicated from the 5'- to 3'-termini. The recombinant DNA thus obtained was named "**pGZ4**". The recombinant DNA pGZ4 had substantially the same structure as the recombinant DNA pGZ3 obtained in Example 6-3 except that it had the nucleotide sequence of SEQ ID NO:8.

The recombinant DNA pGZ4 was introduced into "**BMH71-18mutS**", an *Escherichia coli* competent cell commercialized by Takara Shuzo Co., Ltd., Tokyo, Japan, to obtain a transformant. Using conventional alkali-SDS method, a DNA was extracted from the transformant and confirmed to be pGZ4 according to conventional manner. It was named "**GZ4**". Thus a transformant introduced with a DNA encoding the present trehalose-releasing enzyme.

Example 7

Preparation of trehalose-releasing enzyme

Example 7-1

Preparation of enzyme using microorganisms of the genus *Arthrobacter*

A seed culture of *Arthrobacter* sp. S34, FERM BP-6450, was inoculated to a nutrient culture medium and incubated by a fermenter for about 72 hours in accordance with the method in Example 2-1. After the incubation, the resultant culture was filtered and concentrated with an SF-membrane to obtain an about eight liters of cell suspension which was then treated with "**MINI-LABO**", a super high-pressure cell disrupter commercialized by Dainippon Pharmaceutical Co., Ltd., Tokyo, Japan, to disrupt cells. The cell disruptant was centrifuged to collect and obtain an about 8.5 ℓ supernatant as a cell extract. Determination of the cell extract for trehalose-releasing enzyme activity revealed that the culture contained about 0.3 unit/ml culture of the enzyme activity. To the cell extract was added ammonium sulfate to give a saturation degree of 0.7 to effect salting out, and then centrifuged to obtain the precipitate. The precipitate was dissolved in 10 mM phosphate buffer (pH 7.0), and dialyzed against a fresh preparation of the same buffer. The dialyzed inner solution was subjected to ion-exchange chromatography using "**SEPABEADS FP-DA13 GEL**" commercialized by Mitsubishi Chemical Co., Ltd., Tokyo, Japan, in accordance with the method in Example 5-2 except that the resin volume used of the ion exchanger was about two liters, followed by collecting fractions having an trehalose-releasing enzyme activity. The fractions were pooled and dialyzed against a fresh preparation of the same buffer but containing 1 M ammonium sulfate, and then the dialyzed solution was centrifuged to obtain the formed supernatant. The supernatant was subjected to a hydrophobic column chromatography using "**BUTYL TOYOPEARL**

650M GEL", a hydrophobic gel commercialized by Tosoh Co., Ltd., Tokyo, Japan, in accordance with the method in Example 5-2 except that an about 350 ml of the gel was used, and then fractions with a trehalose-releasing enzyme activity were collected. The enzyme collected was confirmed to have an optimum temperature in a medium temperature range, i.e., temperatures over 45°C but below 60°C and an optimum pH in an acid pH range, i.e., a pH of less than 7.

Thus an about 6,400 units of the present trehalose-releasing enzyme was obtained.

Example 7-2

Preparation of enzyme using microorganism of the genus *Arthrobacter*

A seed culture of *Arthrobacter* sp. S34, FERM BP-6450, was inoculated to a nutrient culture medium in accordance with the method in Example 7-1. To one ℓ of the resulting culture was added 100 mg "**OVALBUMIN LYSOZYME**", a lysozyme preparation, commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan. Then aeration was suspended, and cells were disrupted by keeping the culture for 24 hours under the same temperature and stirring conditions as used in the culture. The cell disruptant was subjected to a continuous centrifuge at 10,000 rpm, followed by collecting a supernatant as a cell extract. In accordance with the method in Example 7-1, the cell extract was treated with salting out, and the sediment was dialyzed. The resulting dialyzed inner solution was subjected to ion-exchange chromatography using "**SEPABEADS FP-DA13 GEL**", a product of Mitsubishi Chemical Co., Ltd., Tokyo, Japan, in accordance with

the method in Example 7-1 to collect fractions with a trehalose-releasing enzyme activity. The pooled fractions contained about 16,500 units of the present trehalose-releasing enzyme and about 5,500 units the present non-reducing saccharide-forming enzyme. Thus an enzyme preparation containing the present two types of enzymes was obtained.

Example 7-3

Production of enzyme using transformant

In a 500-ml Erlenmeyer flask were placed a 100 ml aqueous solution containing 16 g/ℓ polypeptone, 10 g/ℓ yeast extract, and 5 g/ℓ sodium chloride, and the flask was autoclaved at 121°C for 15 min, cooled, aseptically adjusted to pH 7.0, and aseptically admixed with 10 mg ampicillin in a sodium salt to obtained a nutrient culture medium. The transformant "GZ2" obtained in Example 6-2 was inoculated into the liquid medium, followed by the incubation at 37°C for about 20 hours under aeration-agitation conditions to obtain a seed culture. Seven liters of a fresh preparation of the same medium as used in the seed culture were similarly prepared and placed in a 10-ℓ fermenter, inoculated with 70 ml of the seed culture, and cultures for about 20 hours under aeration-agitation conditions. Cells were collected by centrifuging the resulting culture in usual manner. The collected cells were suspended in 10 mM phosphate buffer (pH 7.0) and ultrasonicated to disrupt the cells. The resulting mixture was centrifuged to remove insoluble substances, followed by collecting a supernatant as a cell extract. The cell extract was dialyzed against 10 mM phosphate buffer (pH 7.0). The dialyzed inner solution was

collected and confirmed to have an optimum temperature in a medium temperature range, i.e., temperatures over 45°C but below 60°C and an optimum pH in an acid pH range, i.e., a pH of less than 7.

Thus the present trehalose-releasing enzyme was obtained. In this Example, an about 0.5 unit/ml culture of the trehalose-releasing enzyme was obtained.

As a control, "**XL1-Blue**", an *Escherichia coli* strain commercialized by Stratagene Cloning Systems, California, USA, was cultured under the same culture conditions as used in the above in a fresh preparation of the same culture medium as above but free of ampicillin, followed by collecting and dialyzing a cell extract similarly as above. No trehalose-releasing enzyme activity was observed, meaning that the transformant GZ2 can be advantageously used in producing the present trehalose-releasing enzyme.

Example 7-4

Production of enzyme using transformant

The transformant GZ3 in Example 6-3 was cultured similarly as in Example 7-3 except for using a liquid nutrient culture medium (pH 7.0) consisting of one w/v % maltose, three w/v % polypeptone, one w/v % "**MEAST PIG**" commercialized by Asahi Breweries, Ltd., Tokyo, Japan, 0.1 w/v % dipotassium hydrogen phosphate, 100 μg/ml ampicillin, and water. The resulting culture was treated with ultrasonication to disrupt cells, and the mixture was centrifuged to remove insoluble substances. Measurement of the trehalose-releasing enzyme activity in the resulting supernatant revealed that it contained about 70

units/ml culture of the enzyme. In accordance with the method in Example 5-2, the supernatant was purified and confirmed that the purified specimen had an optimum temperature in a medium temperature range, i.e., temperatures over 45°C but below 60°C and an optimum pH in an acid pH range, i.e., a pH of less than 7. Thus the present trehalose-releasing enzyme was obtained.

Example 7-5

Production of enzyme using transformant

The transformant GZ4 in Example 6-4 was cultured similarly as in Example 4-4. The resulting culture was treated with ultrasonication to disrupt cells, and the mixture was centrifuged to remove insoluble substances. Measurement of the trehalose-releasing enzyme activity in the resulting supernatant revealed that it contained about 250 units/ml culture of the enzyme. In accordance with the method in Example 5-2, the supernatant was purified and confirmed that the purified specimen had an optimum temperature in a medium temperature range, i.e., temperatures over 45°C but below 60°C and an optimum pH in an acid pH range, i.e., a pH of less than 7. Thus the present trehalose-releasing enzyme was obtained.

Example 8

Saccharide production

Example 8-1

Production of non-reducing saccharide syrup

A 6 w/w % potato starch suspension was gelatinized by heating, adjusted to pH 4.5 and 50°C, admixed with 2,500 units/g starch, d.s.b., and enzymatically reacted for 20 hours. The reaction mixture was adjusted to pH 6.5, autoclaved at 120°C for

10 min, cooled to 40°C, admixed with 150 units/g starch, d.s.b., of "**TERMAMYL 60L**", an α-amylase specimen commercialized by Novo Nordisk Industri A/S, Copenhagen, Denmark, and subjected to an enzymatic reaction for 20 hours while keeping at the temperature. The reaction mixture was autoclaved at 120°C for 20 min, cooled to 53°C, adjusted to pH 5.7, admixed with one unit per gram starch, d.s.b., of a non-reducing saccharide-forming enzyme obtained by the method in Example 4-1, and subjected to an enzymatic reaction for 96 hours. The reaction mixture thus obtained was heated at 97°C for 30 min to inactivate the remaining enzyme, cooled, filtered, purified in a usual manner by decoloration with an activated charcoal and desalting with ion exchangers, and concentrated to obtain an about 70 w/w % syrup in a yield of about 90% to the material starch, d.s.b.

The product, which has a low DE of 24 and contains α-glucosyltrehalose, α-maltosyltrehalose, α-maltotriosyltrehalose, α-maltotetraosyltrehalose, and α-maltopentaosyltrehalose in respective amount of 11.5, 5.7, 29.5, 3.5, and 2.8%, d.s.b., has a mild and high-quality sweetness, and a satisfactory viscosity and moisture-retaining ability. It can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, adjuvant or excipient in compositions in general such as foods, cosmetics, and pharmaceuticals.

Example 8-2

Production of syrup containing non-reducing saccharide

To a 33 w/w % corn starch suspension was added calcium carbonate to give a final concentration of 0.1 w/w %, and then

the mixture was adjusted to pH 6.5, admixed with 0.2 w/w % per starch, d.s.b., of "**TERMAMYL 60L**", a liquefying α-amylase specimen commercialized by Novo Nordisk Industri A/S, Copenhagen, Denmark, and enzymatically reacted at 95°C for 15 min to liquefy the starch. The liquefied starch was autoclaved at 120°C for 10 min, cooled to 53°C, admixed with one unit/g starch, d.s.b., of a maltotetraose-forming enzyme from a *Pseudomonas stutzeri* strain commercialized by Hayashibara Biochemical Laboratories Inc., Okayama, Japan, and two units/g starch, d.s.b., of a non-reducing saccharide-forming enzyme obtained by the method in Example 4-2, and enzymatically reacted for 48 hours. The reaction mixture was admixed with 15 units of "**α-AMYLASE 2A**", an α-amylase specimen commercialized by Ueda Chemical Co., Ltd., Osaka, Japan, and then incubated at 65°C for two hours, autoclaved at 120°C for 10 min, and cooled. The resulting mixture was filtered, and in a usual manner purified by treatments of coloration using an activated charcoal and of desalting using ion exchangers, and concentrated into an about 70 w/w % syrup, d.s.b., in a yield of about 90% with respect to the material starch, d.s.b.

The product, which has a low DE of 18.5 and contains α-glucosyltrehalose, α-maltosyltrehalose, α-maltotriosyltrehalose, α-maltotetraosyltrehalose, and α-maltopentaosyltrehalose in respective amount of 9.3, 30.1, 0.9, 0.8, and 0.5%, d.s.b., has a mild and high-quality sweetness, and a satisfactory viscosity and moisture-retaining ability. It can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, adjuvant or

excipient in compositions in general such as foods, cosmetics, and pharmaceuticals.

Example 8-3

Production of non-reducing saccharide

A 20 w/w % aqueous solution of any of reducing partial starch hydrolyzates of maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose, which are all produced by Hayashibara Biochemical Laboratories Inc., Okayama, Japan, admixed with two units/g reducing partial starch hydrolyzate of a purified specimen of non-reducing saccharide-forming enzyme obtained by the method in Example 2-2, and subjected to an enzymatic reaction at 50°C and pH 6.0 for 48 hours. From each of the above-identified reducing partial starch hydrolyzates were respectively formed α-glucosyltrehalose, α-maltosyltrehalose, α-maltotriosyltrehalose, α-maltotetraosyltrehalose, and α-maltopentaosyltrehalose as reducing saccharides. Saccharides in each reaction mixture were in conventional manner fractionated by the following successive treatments: Inactivation of the remaining enzyme by heating, filtration, decoloration, desalting, concentration, and column chromatography using "XT-1016 (Na^+-form)", an alkali-metal strong-acid cation exchange resin with a polymerization degree of 4%, commercialized by Tokyo Organic Chemical Industries, Ltd., Tokyo, Japan. The conditions used in the column chromatography were as follows: The inner column temperature was set to 55°C, the load volume of a saccharide solution to the resin was about 5 v/v %, and the flow rate of water heated to 55°C as a moving bed was set to SV (space velocity) 0.13. An

eluate from each column, which contained at least 95 w/w % of any of the above-identified non-reducing saccharides, d.s.b., with respect to saccharide composition, was collected. To each collected eluate was added sodium hydroxide to give a concentration of 0.1 N, and the mixture was heated at 100°C for two hours to decompose the remaining reducing saccharides. The reaction mixtures thus obtained were respectively decolored with an activated charcoal, desalted with ion exchangers in H- and OH-form, concentrated, dried *in vacuo*, and pulverized into powdery α-glucosyltrehalose, α-maltosyltrehalose, α-maltotriosyltrehalose, α-maltotetraosyltrehalose, and α-maltopentaosyltrehalose with a purity of at least 99.0 w/w %, d.s.b.

The products, containing highly-purified non-reducing saccharides and having a more lower DE, can be arbitrarily used as a taste-improving agent, quality-improving agent, stabilizer, filler, adjuvant or excipient in compositions in general such as foods, cosmetics, and pharmaceuticals.

Example 8-4

Production of crystalline powder containing non-reducing saccharide

An aqueous 20 w/w % solution of maltopentaose commercialized by Hayashibara Biochemical Laboratories Inc., Okayama, Japan, was prepared, admixed with two units/g maltopentaose, d.s.b., of a non-reducing saccharide-forming enzyme obtained by the method in Example 4-3, and enzymatically reacted at 50°C for 48 hours, resulting in a conversion of about 75% maltopentaose into α-maltotriosyltrehalose. The reaction

mixture was heated at 97°C for 30 min to inactivate the remaining enzyme, and then cooled, filtered, and purified by decoloration using an activated charcoal and desalting using ion exchangers.

Thereafter, the resulting solution was concentrated into an about 75 w/w % solution with respect to solid contents, admixed with an about 0.01 w/v α-maltotriosyltrehalose crystal as a seed crystal, and allowed to stand for 24 hours. Then the crystallized α-maltotriosyltrehalose crystal was collected by a centrifuge, washed with a small amount of cold water, and dried in a usual manner to obtain a crystalline powder with a relatively-high content of the non-reducing saccharide in a yield of about 50% to the material solids, d.s.b.

The product, having a relatively-low sweetness and an extremely-low DE of less than 0.2 and containing at least 99.0 w/w % of α-maltotriosyltrehalose as a non-reducing saccharide, can be arbitrarily used as a taste-improving agent, quality-improving agent, stabilizer, filler, adjuvant or excipient in compositions in general such as foods, cosmetics, and pharmaceuticals.

Example 8-5

Process for producing hydrous crystalline trehalose

Corn starch was suspended in water into a 30 w/w % starch suspension which was then admixed with calcium carbonate in an amount of 0.1 w/w %. The mixture was adjusted to pH 6.0, and then admixed with 0.2 w/w % per starch, d.s.b., of "**TERMAMYL 60L**", a liquefying α-amylase specimen commercialized by Novo Nordisk Industri A/S, Copenhagen, Denmark, and enzymatically

reacted at 95°C for 15 min to gelatinize and liquefy the starch. The resulting mixture was autoclaved at 120°C for 30 min, cooled to 51°C, adjusted to pH 5.7, and enzymatically reacted at the same temperature for 64 hours after admixed with 300 units/g starch, d.s.b., of an isoamylase specimen commercialized by Hayashibara Biochemical Laboratories Inc., Okayama, Japan; two units/g starch, d.s.b., of a cyclomaltodextrin glucanotransferase specimen commercialized by Hayashibara Biochemical Laboratories Inc., Okayama, Japan; two units of a non-reducing saccharide-forming enzyme obtained by the method in Example 4-1; and 10 unit/g starch, d.s.b., of a trehalose-releasing enzyme obtained by the method in Example 7-1. The reaction mixture was heated at 97°C for 30 min to inactivate the remaining enzyme, and then cooled 50°C, admixed with 10 unit/g starch, d.s.b., of "**GLUCOZYME**", a glucoamylase specimen commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and subjected to an enzymatic reaction for 24 hours. The reaction mixture thus obtained was heated at 95°C for 10 min to inactivate the remaining enzymes, cooled, filtered, purified by decoloration using an activated charcoal and desalting using ion exchangers, and concentrated to an about 60 w/w % solution with respect to solid contents or a syrup containing 84.1 w/w % trehalose, d.s.b. The syrup was concentrated up to give a concentration of about 83 w/w %, d.s.b., and the concentrate was placed in a crystallizer, admixed with an about 0.1 w/v % hydrous crystalline trehalose to the syrup, and stirred for about two hours to crystallize the saccharide. The resulting crystals were collected by a centrifuge, washed with a small

amount of water to remove molasses, dried by air heated to 45°C to obtain hydrous crystalline trehalose with a purity of at least 99% in a yield of about 50% to the material starch, d.s.b.

Since the product is substantially free from hygroscopicity and easily handleable, it can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, adjuvant or excipient in compositions in general such as foods, cosmetics, and pharmaceuticals.

Example 8-6

Process for producing crystalline powder containing anhydrous crystalline trehalose

Using the method in Example 8-5 hydrous crystalline trehalose was prepared, and the saccharide was dried *in vacuo* using a jacketed rotary-vacuum-dryer. The drying was conducted at 90°C and 300-350 mmHg for about seven hours. After the drying, the above temperature and pressure were returned to ambient temperature and normal pressure before collecting the product or a crystalline powder containing at least 90 w/w % anhydrous crystalline trehalose, d.s.b.

Since anhydrous crystalline trehalose absorbs moisture in hydrous matters and changes in itself into hydrous crystalline trehalose, the product rich in the saccharide can be arbitrarily used as a non-harmful safe desiccant to dehydrate or dry compositions including food products, cosmetics and pharmaceuticals, as well as materials and intermediates thereof. The product with a mild and high-quality sweetness can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, adjuvant or excipient in

compositions in general such as foods, cosmetics, and pharmaceuticals.

Example 8-7

Process for producing trehalose syrup

A 27 w/w % suspension of tapioca starch was admixed with calcium carbonate to give a final concentration of 0.1 w/w %, adjusted to pH 6.0, admixed with 0.2 w/w % per starch, d.s.b., of "**TERMAMYL 60L**", a liquefying α-amylase specimen commercialized by Novo Nordisk Industri A/S, Copenhagen, Denmark, and enzymatically reacted at 95°C for 15 min to gelatinize and liquefy the starch. The resulting mixture was autoclaved at a pressure of 2 kg/cm^2 for 30 min, cooled to 53°C, adjusted to pH 5.7, and enzymatically reacted at the same temperature for 72 hours after admixed with 500 units/g starch, d.s.b., of "**PROMOZYME 200L**", a pullulanase specimen commercialized by Novo Nordisk Industri A/S, Copenhagen, Denmark; one unit/g starch, d.s.b., of *Pseudomonas stutzeri* strain commercialized by Hayashibara Biochemical Laboratories Inc., Okayama, Japan; about two units/g starch, d.s.b., of a non-reducing saccharide-forming enzyme and about six units/g starch, d.s.b., of a trehalose-releasing enzyme, obtained by the method in Example 7-2. The reaction mixture thus obtained was heated at 97°C for 15 min, cooled and filtered to obtain a filtrate. The filtrate was in a usual manner purified by decoloration using an activated charcoal and desalting using ion exchangers, and concentrated to an about 70 w/w % syrup with respect to solid contents in a yield of about 92% to the material, d.s.b.

The product, comprising 35.2% trehalose, 3.4% α-glucosyltrehalose, 1.8% glucose, 37.2% maltose, 9.1% maltotriose, and 13.3% oligosaccharides higher than maltotetraose, has a mild and high-quality sweetness, relatively-lower reducibility and viscosity, and adequate moisture-retaining ability; it can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, adjuvant or excipient in compositions in general such as foods, cosmetics, and pharmaceuticals.

Example 8-8

Process for producing crystalline powder containing anhydrous crystalline trehalose

One part by weight of "**EX-I**", an amylose commercialized by Hayashibara Biochemical Laboratories Inc., Okayama, Japan, was dissolved in 15 parts by weight of water by heating, and the solution was heated to 53°C and adjusted to pH 5.7. To the resulting solution was added two units/g amylose, d.s.b., of a non-reducing saccharide-forming enzyme, obtained in Example 4-3, and six units/g amylose, d.s.b., of a trehalose-releasing enzyme, obtained by the method in Example 7-4, followed by an incubation for 48 hours. The reaction mixture was heated at 97°C for 30 min to inactivate the remaining enzyme, and then adjusted to pH 5.0, admixed with 10 units/g amylose, d.s.b., of "**GLUCOZYME**", a glucoamylase specimen commercialized by Nagase Biochemicals, Ltd., Kyoto, and enzymatically reacted for 40 hours. The reaction mixture thus obtained was heated at 95°C for 10 min to inactivate the remaining enzymes, cooled, filtered, purified by decoloration

using an activated charcoal and desalting using ion exchangers, and concentrated to an about 60 w/w % solution with respect to solid contents or a syrup containing 82.1 w/w % trehalose, d.s.b.

Similarly as in Example 8-3, the syrup was subjected to column chromatography, followed by collecting a fraction containing about 98 w/w % trehalose, d.s.b. The fraction was concentrated *in vacuo* under heating conditions into an about 85 w/w % syrup with respect to solid contents. The syrup was admixed with hydrous crystalline trehalose as a seed crystal in an about 2 w/v % of to the syrup, stirred at 120°C for five minutes, distributed to plastic vats, and dried at 100°C *in vacuo* to crystallize the saccharide. Thereafter, the contents in a block form were detached from the vats and cut with a cutter to obtain a solid product, containing anhydrous crystalline trehalose with a crystallinity of about 70% and having a moisture content of about 0.3 w/w % in a yield of about 70% to the material amylose, d.s.b. The solid product was pulverized in a usual manner into a crystalline powdery containing anhydrous crystalline trehalose.

Since anhydrous crystalline trehalose absorb moisture from hydrous matters and changes into hydrous crystalline trehalose, the product rich in anhydrous crystalline trehalose can be arbitrarily used as a non-harmful safe desiccant to dehydrate or dry compositions including food products, cosmetics and pharmaceuticals, as well as materials and intermediates thereof. The product with a mild and high-quality sweetness can be arbitrarily used as a sweetener, taste-improving agent,

quality-improving agent, stabilizer, filler, adjuvant or excipient in compositions in general such as foods, cosmetics, and pharmaceuticals.

As described above, the present invention was made based on the finding of a novel non-reducing saccharide-forming enzyme and a novel trehalose-releasing enzyme, which have an optimum temperature in a medium temperature range and preferably have an optimum pH in an acid pH range. These enzymes according to the present invention can be obtained in a desired amount, for example, by culturing microorganisms capable of producing the enzymes. The present DNAs which encode either of the enzymes are quite useful in producing such enzymes as recombinant proteins. In cases of using transformant introduced with the DNAs, the enzymes according to the present invention can be yielded in a desired amount. The present enzymes can be used in producing non-reducing saccharides having a trehalose structure, which include trehalose, in a medium temperature rang and/or an acid pH range. Particularly, when used the present enzymes in combination with other saccharide-related enzymes having an optimum temperature in a medium temperature rang and/or an optimum pH in an acid pH range, desired saccharides can be produced quite efficiently. The enzymes according to the present invention are ones with revealed amino acid sequences; they can be safely used to produce the non-reducing saccharides to be used in food products and pharmaceuticals. The non-reducing saccharides and reducing saccharides, which contain the same and have a lesser reducibility, produced by the present invention have a mild and high-quality sweetness, and most

preferably have an insubstantial reducibility or a reduced reducibility by a large margin. Therefore, the saccharides can be arbitrarily used as in compositions in general such as foods, cosmetics, and pharmaceuticals with lesser fear of coloration and deterioration.

The present invention with these unfathomable advantageous properties and features is a useful invention that would greatly contribute to this art.

While there has been described what is at present considered to be the preferred embodiments of the invention, it will be understood that various modifications may be made therein, and it is intended to cover in the appended claims all such modifications as fall within the true spirit and scope of the invention.